Amphiphilic Starch -

A New Excipient for Ophthalmic Application

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina

zu Braunschweig

zur Erlangung des Grades einer

Doktorin der Naturwissenschaften

(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n

von Luma Baydoun
aus Braunschweig

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eingereicht am: 19.09.2007
mündliche Prüfung (Disputation) am: 25.02.2008

Druckjahr 2008

Für meine Familie

Die vorliegende Arbeit entstand unter der Leitung von Frau Professor Dr. Christel Müller-Goymann am Institut für Pharmazeutische Technologie der Technischen Universität Carolo-Wilhelmina zu Braunschweig.

Ich danke Frau Professor Dr. Christel Müller-Goymann herzlich für die Aufnahme in ihre Arbeitsgruppe, die Überlassung des Themas und die damit verbundene Ermöglichung im Fach Technologie wertvolle Erfahrungen zu sammeln. Vielen Dank für die freundliche Betreuung und persönliche Begleitung.

Ich danke

allen technischen Mitarbeitern des Instituts, insbesondere Martha Vasquez-Borbe, Kirsten Nebelung, Juliane Schildt und Ursula Jahn, für die Durchführung zahlreicher Messungen und die stets gute Zusammenarbeit. Es hat sehr viel Spaß gemacht, mit Euch zu arbeiten.

allen Hilfswissenschaftlern, Studenten und Doktoranden des Instituts sowie Mitarbeitern anderer Institute, insbesondere Herrn Dr. Vieregge vom Institut für Pharmakologie und Toxikologie, für die angenehme Zusammenarbeit und das kollegiale Arbeitsklima. An dieser Stelle gilt mein herzlichster Dank auch Herrn Professor Dr. Claus Führer und Herrn Professor Dr. Rolf Daniels.

Frau Professor Dr. Annick Ludwig für die Anfertigung des Zweitgutachtens und die freundliche Aufnahme am Institut für „Farmaceutische Technologie“ der Universität Instellings, Antwerpen, zur Durchführung rheologischer und oculär-fluorophotometrischer Untersuchungen sowie die vielen wertvollen Anregungen. Meinen Kollegen Dr. Wim Weyenberg, Dr. Jens Ceulemans und Dr. Jo Vandervoort danke ich für die ständige Diskussionsbereitschaft sowie die freundschaftliche Arbeitsatmosphäre.

Herrn Prof. Robert Gurny für die freundliche Aufnahme am „Institut de Chimie Thérapeutique“ der Universität Lausanne zur Durchführung toxikologischer Untersuchungen am Auge und den Mitarbeitern des Institiuts für die freundliche Kooperation. Besonders danke ich Herrn Dr. Pascal Furrer für die umfangreiche Einweisung in die CLSO-Messungen.

Frau Katharina Vogel und Herrn Dr. Kupsch vom Institut für Pathologie, Celler Straße (Braunschweig) für die Anfertigung histologischer Schnitte und die Anleitung bei der pathologischen Befundung.

Herrn Dr. Friedrich Heinze (National Starch & Chemical) und Herrn Dr. Olaf Häusler (Roquette Frères) und Ciba-Geigy/Novartis für die freundliche Überlassung von Materialien.

Herrn Arnold Eilmes (Bundesforschungsanstalt für Landwirtschaft, Braunschweig), Herrn Adolf Düvel (Fleischerei Düvel, Lauenau) und der Fleischerei Jahns (Hornburg) für die freundliche Überlassung von Schweineaugen.

Mein besonderer Dank gilt Herrn Dr. Muhannad Jumaa für die inspirierende „Starthilfe“.

Ich danke meiner Schwester Lamis und meiner lieben Kollegin und Freundin Dr. Fadwa Hussein für die Unterstützung, die Durchsicht der Arbeit und die konstruktive und hilfreiche Kritik.

Ich danke meiner Schwester Mariam, dass sie so viel Zeit mit Iman verbracht hat. Das war eine große Hilfe. Danke auch für die unendlich „positive Energie“ während der Prüfungsvorbereitungen.

Ich danke meinen lieben Eltern für ihren bedingungslosen Beistand, ihren ansteckenden Optimismus und dass sie mir meinen größten Wunsch, Pharmazie zu studieren, ermöglicht haben.

Hussam, danke, dass ich mich immer auf Dich verlassen kann.

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch die Mentorin der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Publikationen

Baydoun, L. & Müller Goymann C. C. Influence of n-octenylsuccinate starch on in vitro permeation of sodium diclofenac across excised porcine cornea in comparison to Voltaren ophtha. European Journal of Pharmaceutics and Biopharmaceutics 56(1): 73-79 (2003).

Baydoun L., Furrer P., Gurny R., Müller-Goymann C. C. New surface-active polymers for ophthalmic formulations: evaluation of ocular tolerance. European Journal of Pharmaceutics and Biopharmaceutics 58(1): 169-175 (2004).

Tagungsbeiträge

Baydoun, L. & Müller-Goymann C. C. Amphiphilic starch as a new excipient for pharmaceutical applications (Poster), 3rd World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Berlin (2000)

Baydoun, L. & Müller-Goymann C. C. Amphiphilic starch: a stabilising agent for medicinal emulsions (Poster), Jahrestagung der Deutschen Pharmazeutischen Gesellschaft (DPhG), Halle/Saale (2001)

Baydoun, L., Ludwig A. & Müller-Goymann C. C. Influence of amphiphilic starch on in vitro permeation of diclofenac sodium through porcine cornea and investigation of interaction with mucin (Vortrag), 4th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology , Florenz/Italien (2002)

Baydoun, L. & Müller-Goymann C. C. Untersuchung des cornealen Permeations-verhaltens von Diclofenac-Natrium aus verschiedenen Zubereitungen im Vergleich

zu Voltaren ophtha (Vortrag), Jahrestagung der Deutschen Pharmazeutischen Gesellschaft (DPhG), Berlin (2002)

Baydoun L., Furrer P., Gurny R., Müller-Goymann C. C. New surface-active polymers for ophthalmic formulations: evaluation of ocular tolerance (Poster), International Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Nürnberg (2004)

Baydoun, L., Ludwig A. & Müller-Goymann C. C. Development of an *n*-octenylsuccinate starch formulation with prolonged precorneal residence time: fluorophotometric investigation of ocular kinetics (Poster), International Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Genf/Schweiz (2006)

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Intent and preview of the investigations

According to the Food and Drug Administration (FDA) pharmaceutical excipients are any inactive ingredients that are intentionally added to therapeutic and diagnostic products, but that are not intended to exert therapeutic effects at the intended dosage. Nevertheless excipients may improve drug delivery, such as enhance absorption or control drug release [Food and Drug Administration, 2005]. The term “new excipients” refers to ingredients that are not fully qualified by existing safety data with respect to the currently proposed level of exposure, duration of exposure, or route of administration [Food and Drug Administration, 2005].

The excipient *n*-octenylsuccinate starch (Fig. 1), further referred to as AS, is an FDA approved food and cosmetics additive with amphiphilic characteristics. Starch is chemically modified by replacing hydrophilic starch moieties by lipophilic *n*-octenylsuccinic acid groups [Caldwell and Wurzburg, 1953; Järnström et al., 1995]. AS sodium salts especially perform as stabilizers for emulsions and as encapsulation carriers for lipophilic or oxidizable substances.

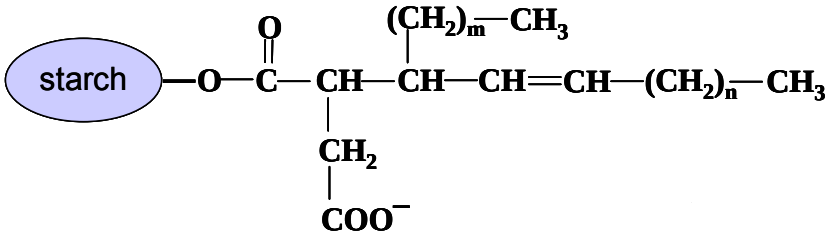

Fig. 1: Chemical structure of *n*-octenylsuccinate starch (m = 1 and n = 2 or m = 2 and n = 1)

Though at present not approved for pharmaceutical applications, a few publications deal with investigations on AS employed in drug dosage forms, serving as a dissolution enhancer in solid dosage forms [Ntawukuliyayo et al., 1993] or carrier in hydrophilic sustained release matrices [Ntawukuliyayo et al., 1996].

The primary aim of this thesis was to assess whether AS is an applicable excipient for non-enteral therapeutics with focus on ophthalmic formulations. Sodium diclofenac (DfNa), a therapeutic in ocular inflammation treatment and associated with

problems such as instability in aqueous solutions and low corneal permeation rates, was chosen as a model drug. Advantages and limitations of AS in drug-loaded and unloaded systems were evaluated, regarding pharmaceutical technological and biopharmaceutical aspects and toxicology.

PART I – Pharmaceutical technological aspects

Since emulsions are suitable carrier systems for DfNa [Bock et al., 1994] the capability of AS as an oil-in-water emulsion stabilizer was studied. In chapter 2 two AS types with different molecular weights were assessed for their emulsifying properties.

Before incorporating the model drug substance sodium diclofenac into AS carrier systems drug stability and drug-excipient interaction studies were performed using isothermal heat conduction calorimetry, differential scanning calorimetry and HPLC (chapter 3).

The AS type, that proved to be more suitable, as drawn from results obtained in chapter 2, was further employed (chapter 4) to develop emulsions with appropriate composition, particle size distributions and long-term stability. The influence of processing parameters of high pressure homogenization and preparation, e. g. spray drying, emulsion composition, using different oils and co-emulsifiers, and pH variations on emulsion formation and stability during storage and autoclaving were studied. Excipient interactions were measured by isothermal heat conduction calorimetry.

PART II – Cytotoxicity studies and ocular tolerance

Surface active additives in commercial ophthalmic preparations may act as solubilizers for poorly soluble drugs, emulsion/suspension stabilizers or penetration enhancers. However, these substances have to be chosen and dosed carefully since

they are often irritating causing a rapid removal of the therapeutic from the site of absorption.

Chapter 6 deals with the evaluation of the irritation potential of different AS types and formulations. In vitro tissue acceptability studies on human erythrocytes and excised porcine cornea and in vivo ocular tolerance tests were carried out in rabbit eyes using confocal laser scanning microscopy.

PART III – Biopharmaceutical aspects

Numerous protective mechanisms of the eye including reflex blinking, lachrymation and a highly selective corneal barrier make the development of ocular dosage forms, which enhance corneal permeation and prolong the drug's precorneal residence time, an interesting challenge. Mucoadhesive substances can be added to ocular drug delivery systems to improve ocular residence time and bioavailability. While in chapter 8 the influence of AS on the in vitro corneal permeation behaviour of sodium diclofenac is discussed, in chapter 9 the interaction of AS and mucin was investigated rheologically. Finally chapter 10 deals with the in vivo evaluation of the pre-ocular residence time of different AS formulations labelled with sodium fluorescein and its elimination from the anterior chamber compartment using ocular fluorophotometry in human volunteers.

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Järnström L, Lason L, Rigdahl M, Erikson U. 1995. Flocculation in kaolin suspensions induced by modified starches II. Oxidized and hydrophobically modified oxidized starch – in comparison with poly(viny alcohol) and carboxymethylcellulose. Colloids and surfaces. 104: 207 – 16

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Part I

Pharmaceutical technological aspects

1 *n*-octenylsuccinate starch and general aspects of emulsion formulation

1.1 *n*-octenylsuccinate starch

Native starch granules are primarily composed of two polyglucans, amylose and amylopectin. Amylose, which is a linear polymer, consists of 1,4-linked α-D-glucopyranosyl units. Amylopectin is a branched polymer of 1,4-linked α-D-glucopyranosyl units with branches resulting from 1,6-linkages. Within the granule, amylopectin is partially crystalline, while amylose is completely amorphous [Shannon and Garwood, 1984].

Derivatization of starch is conducted to modify typical starch properties like gelatinization, cooking characteristics or decrease set-back tendencies of amylose containing starch granules. Waxy maize starch has an amylopectin content of almost 99 %. Since amylopectin requires much longer to set back than amylose, waxy maize starch pastes remain fluid on cooling. Therefore waxy maize starch is used for most food applications [Moore, 1984; Viswanathan 1999a].

Hence, modification is an important factor providing appropriate starch products with defined thickening, swelling, gelling, binding or adhesive qualities to meet the formulator's needs. Starch derivatives include those modifications which change the chemical structure of the D-glucopyranosyl molecule, usually involving oxidation, esterification, etherification, hydrolysis and dextrinization [Rutenberg and Solarek, 1984].

Due to its polyhydroxyl groups, starch is hydrophilic and therefore incompatible with water-insoluble substances. Modification by adding lipophilic groups leads to amphiphilic properties. Hydrophobically modified starches can be obtained by reaction of starch with alkylene oxides which are most commonly hydroxyethyl or hydroxypropyl chains. While the pulp and paper industry prefers products with a low degree of substitution (ds) highly substituted products may serve as hydrophobic coatings or adhesives [Rutenberg and Solarek, 1984; Wesslén and Wesslén, 2002]. Medical applications of hydroxyethylstarch cover its use as a blood volume extender or as a cryoprotective agent for erythrocytes [Rutenberg and Solarek, 1984].

Amphiphilic starch of the *n*-octenylsuccinate starch type (AS), with a molecular weight over 5000 D, is a chemically modified waxy maize starch gained by substituting hydrophilic starch moieties by lipophilic *n*-octenylsuccinic acid groups (Fig. 1-1) [Caldwell and Wurzburg, 1953; Järnström et al., 1995; Gers-Barlag and Müller, 2002]. Consequently, the starch acquires emulsifying properties due to feasible formation of hydrophobic-hydrophobic interactions.

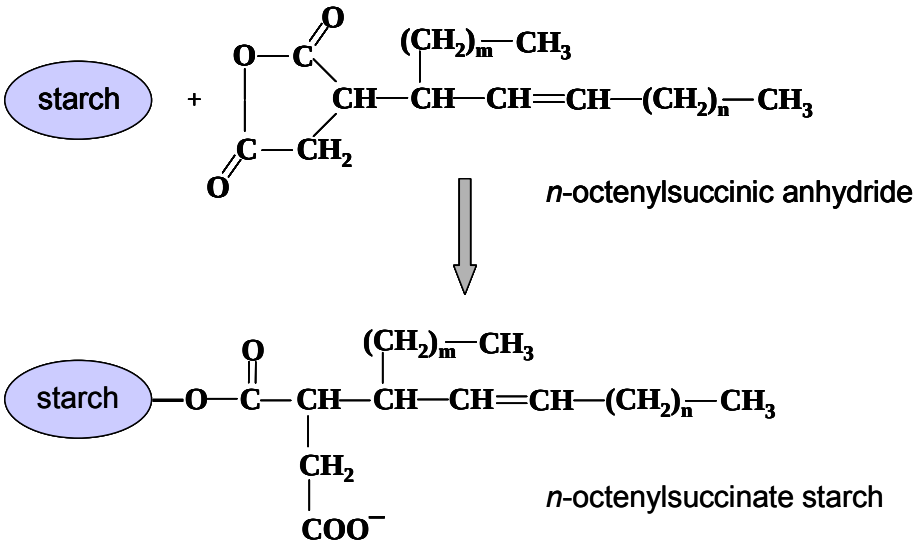

Fig. 1-1: Esterification of starch with cyclic octenylsuccinic anhydride (m = 1 and n = 2 or m = 2 and n = 1)

AS is an FDA-approved food additive with the stipulation that the octenylsuccinate content does not exceed 3 % [Food and drug administration, 1989; Park and Goins, 1995]. Due to emulsifying properties and high retention of volatiles, the main application field of AS is the food and cosmetic industry. AS is an economical alternative to traditional stabilizers such as spray dried gum acacia, vegetal proteins, lecithin or gelatin which are associated with problems of uncertain supply and fluctuating price [Järnström et al., 1995; McGlinchey, 1997]. AS sodium salts especially perform as stabilizers for beverage emulsions or dressings and as encapsulation carriers for oxidizable substances useful in nutritional supplementation [King and Perry, 1976; Buttolph and Newberne, 1979; Järnström et al., 1995]. AS aluminium salts on the other hand are highly hydrophobic [Nair and Yamarik, 2002] and can be used as a talc replacement in body powders to adsorb moisture or excess oil from the skin.

Since a high swelling capacity and high viscosity are not in accordance with the formulators' requirements, additional treatment of fluidification is necessary. By dextrinization, acid or enzymatic hydrolysis or extrusion, a range of octenylsuccinated starches with different levels of viscosity is available. AS is highly dispersible in cold water and produces low viscosities at high solid contents [Heinze, 2000], which is a major point in encapsulation technology.

Despite the modification, biodegradability of the starch is maintained [Järnström et al., 1995]. AS has proven to be useful in the development of degradable plastics composed of AS and hydrophobic plastics [Evangelista et al., 1991; Jane et al., 1991]. However, substituted starch shows higher resistance to enzymatic degradation [Viswanathan, 1999b]. Test persons have shown a reduced glycemic response after AS intake with food as compared to an equivalent glucose challenge. Thus, food containing AS has a decreased caloric density and may perform well as a carbohydrate source with higher glycemic control [Wolf et al., 2001]. Furthermore, it has been found that increasing the ds of AS does not necessarily improve emulsification activity [Järnström et al., 1995].

AS may be beneficial for certain aspects of pharmaceutical formulation. As reported in the literature, low substituted AS increases dissolution rates in solid dosage forms [Ntawukuliyayo et al., 1993]. Associations of AS with xanthan-gum have revealed advantages in the formulation of hydrophilic sustained release matrices [Ntawukuliyayo et al., 1996a] and properties of crystal growth inhibition in sucrose ester suspensions [Ntawukuliyayo et al., 1996b].

1.2 Emulsions as carrier systems for sodium diclofenac

Diclofenac (Fig. 1-2), a non-steroidal anti-inflammatory drug (NSAID), is a widely spread active substance in solid dosage forms [Al Gohary, 1998]. In eye drop formulations NSAIDs are used as an alternative to topical steroids in the treatment of (postoperative) ocular inflammation [Agata et al., 1984; Goodman and Gilman, 1996; González-Peñas et al., 1998].

Due to its low solubility diclofenac (Df) is commercially available as sodium diclofenac (DfNa) [Palomo et al., 1999]. Aqueous DfNa solutions are chemically and physically unstable [Backensfeld et al., 1991]. Considering parenteral and ophthalmic DfNa preparations this problem is overcome by the addition of antioxidants, stabilizing agents, such as polyoxyethylene castor oil as found in the commercial eye drop formulation Voltaren ophtha, or cyclodextrins [Backensfeld et al., 1991]. As reported, DfNa solubility in aqueous systems can also be increased [Baydoun and Müller-Goymann, 2003] with octenylsuccinate starch (AS).

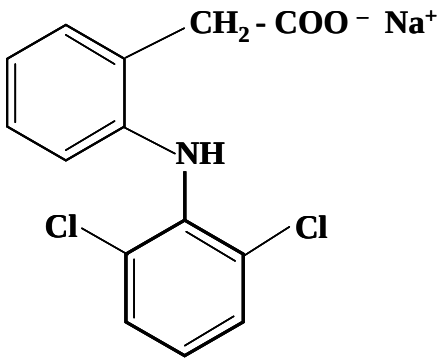

Fig. 1-2: Chemical structure of sodium diclofenac (DfNa)

At physiological pH values Df is mainly charged, therefore polar with a weaker membrane permeability. Decreasing the formulation's pH to improve membrane permeability, in turn affects the drug's solubility. As reported in the literature emulsions are suitable carrier systems for DfNa [Bock et al., 1994]. Emulsions can solubilize considerable amounts of amphiphilic or lipophilic drugs, with partition coefficients higher than 1, in the surface monolayer or within the lipophilic core. Diclofenac is a weak acid (pK_a 4.0) with a partition coefficient of 13 in octanol/phosphate buffer (pH 7.4) [Khazaeinia and Jamali, 2003]. Therefore, in an emulsion system Df is likely to be located in the lipophilic core of the oil droplets [Lindenstruth and Müller, 2004] or, due to its amphiphlic character, in the interface [Reer, 1994].

Appropriate emulsions for ocular or parenteral application, stabilized, e.g. with phospholipids [Reer, 1994] and a non-ionic ABA copolymer surfactant [Jumaa and Müller, 1998], can be easiliy autoclaved and they may decrease the irritating

potential of irritative substances [Jumaa and Müller, 2000]. Furthermore, as compared to eye drop solutions emulsions may show an extended ocular residence time due to a higher viscosity.

1.3 Isothermal heat conduction calorimetry: a tool to investigate drug stability and excipient compatibility

Information about interaction potential are crucial in preformulation studies with new excipients since certain drug-excipient interactions can change stability and bioavailability of a product [Al Gohary, 1998]. In order to assess information on compatibility and stability of drugs in combination with excipients different methods, like differential scanning calorimetry (DSC) [Al Gohary, 1998; Palomo et al., 1999] and isothermal heat conduction calorimetry (IHCC), using a thermal activity monitor (TAM), can be applied [Selzer et al., 1998]. Although thermal analyses do not replace chemical methods, such as HPLC, valuable information about degree of interaction and change in characteristic features of a drug can be obtained.

The thermal activity monitor (TAM) (Fig. 1-3) is a multichannel microcalorimetric system to measure several samples simultaneously, depending on the number of channels the apparatus is equipped with. IHCC can be used to investigate stability, compatibility, amorphicity, polymorphism, binding and many other characteristics of compounds that are important to the pharmaceutical industry. Isothermal microcalorimetry is a technique by which the heat flow produced by a chemical, physical or biological process is continuously monitored while the sample is kept at isothermal conditions. The heat flow is directly related to the rate of heat produced or consumed by the sample placed in the calorimeter. In most cases, the sample is contained in a removable insertion vessel positioned in an ampoule holder inside the microcalorimeter during measurement [Suurkuusk and Wadsö, 1982].

Calorimetric sample vessels can be classified as closed and open ampoules. A closed ampoule is totally sealed from the environment and is generally employed when studying slow processes such as in stability or compatibility experiments. A special case of a closed ampoule is having a solid sample and a saturated salt

solution or pure water, physically separated but connected through the vapour phase inside the ampoule, to create defined humidities. After a sorption process a physical or chemical reaction of the sample takes place, e.g. a slow decomposition reaction, solvate formation or recrystallization of amorphous lactose [Suurkuusk and Wadsö, 1982].

Open ampoules are used when a process is to be initiated in situ after a base line has been recorded. An example is alteration of the chemical composition of a solution by injecting a catalyst, reactant or a ligand in order to initiate a physical, chemical or biological response, which is then continuously measured [Suurkuusk and Wadsö, 1982].

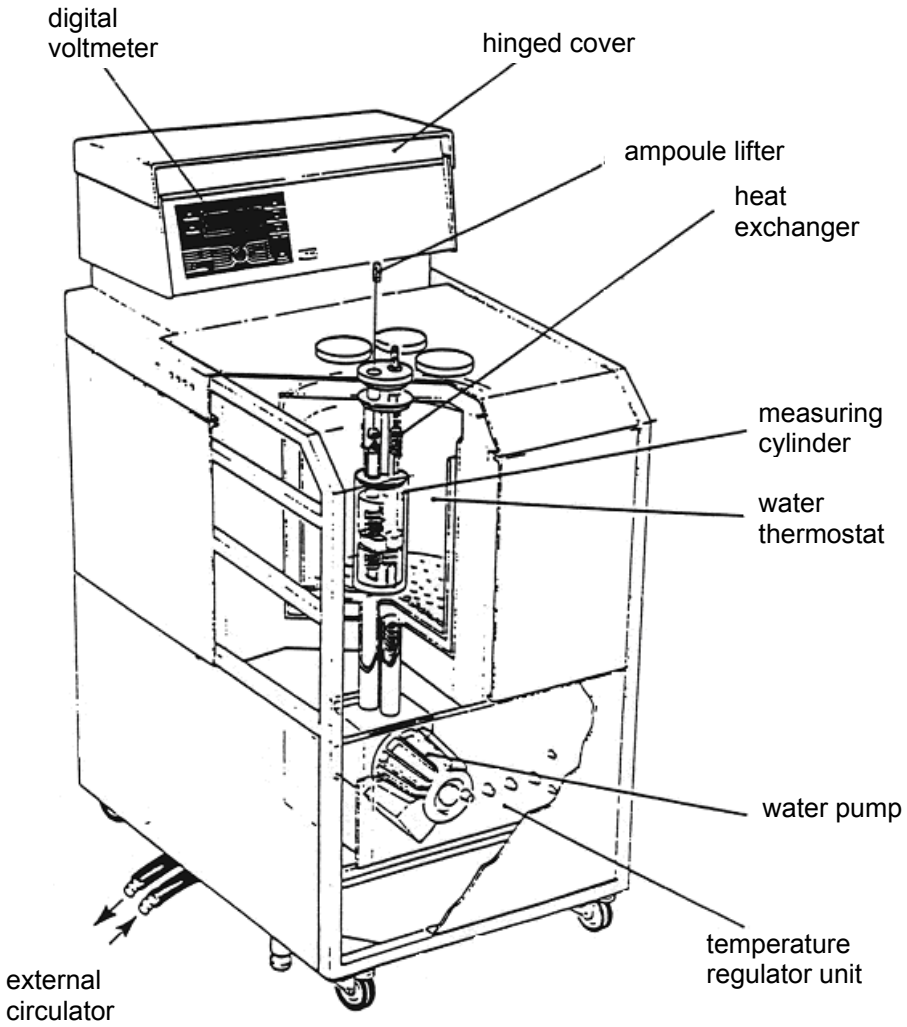

Fig. 1-3: Schematic assembly of a thermal activity monitor (TAM 2277, Thermometric, Jarfälla, Sweden)

Stability can refer to both chemical and physical stability of a raw drug or formulation.

At present, the standard method to study stability of a chemical substance is HPLC. The change in the concentration of the substance itself or its decomposition product(s) is measured as a function of storage time. However, the sensitivity of HPLC is limited. The application of IHCC in stability studies, which has proven to be more sensitive than HPLC [Beezer et al., 1998; Phipps and Mackin, 2000], offers the advantages of being non-invasive, the samples do not need further preparation prior to analysis, such as proper dissolution, and formulated dosage forms can directly be investigated [Beezer et al., 1998; Selzer et al, 1998]. Nevertheless, calorimetry is a non-specific technique and signals not always simply correspond to chemical decomposition but may be a complex signal of thermal events, such as drying, sorption, melting, change in crystallinity, etc. [Lehto and Laine, 1997; Selzer et al., 1998; Phipps and Mackin, 2000].

Compatibility is an important area in the drug and dosage form development. Conventional compatibility studies require both multiple sample preparation and long storage periods to obtain meaningful results. Especially larvated incompatibilities, which cannot be detected macroscopically directly after formulation, can often only be identified during long time storage. Using IHCC an easy method can be applied to predict and study compatibility of binary mixtures within a shorter time [Phipps and Mackin, 2000].

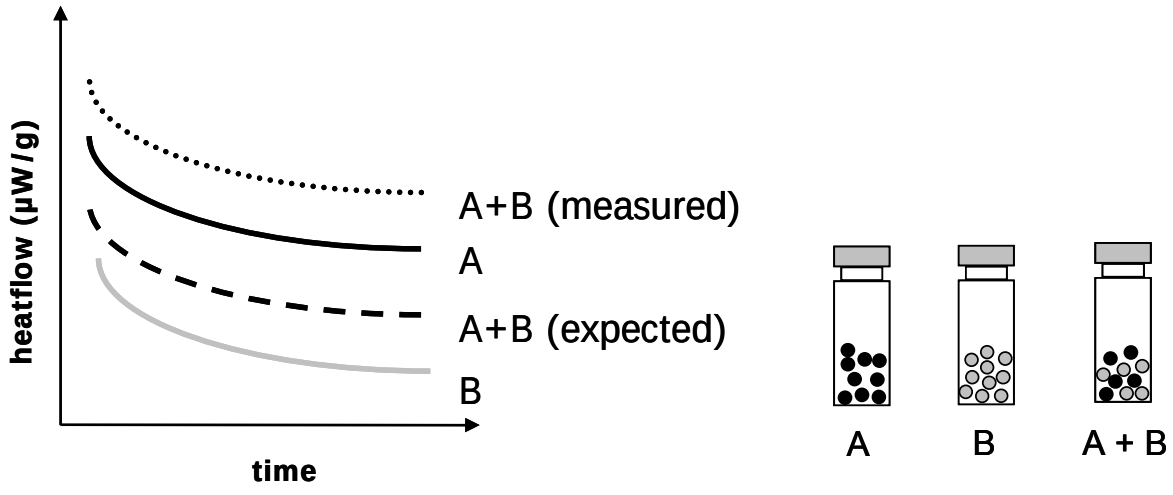

Fig. 1-4: Calorimetric responses (exemplified) of two incompatible substances (A and B): the measured mixture profile (A + B (measured)) differs from the calculated/expected mixture profile (A + B (expected)).

The method involves, after measuring the two substances separately (Fig. 1-4, A, B) in the microcalorimeter under defined conditions (temperature, relative humidity),

preparing a corresponding binary mixture (Fig. 1-4, A + B), which is measured under the same conditions. As exemplified in Figure 1-4 the mixture presented is clearly incompatible as the mixture profile (A + B (measured)) is very different from the profile calculated (A + B (expected)) from the individual components' curves (A, B).

The microcalorimetric method is only designed as a screen and, according to stability studies, serves as a complementary method to conventional analysis. It does not give quantification of the amount of degraded active or degree of interaction. The amount of heat flow released from the mixture sample provides valuable information as to which excipients are likely to be compatible and allows cutting down the number of conventional compatibility samples to prepare and saving time [Phipps and Mackin, 2000].

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2 Comparison of two different *n*-octenylsuccinate starch types with respect to emulsifying properties

2.1 Introduction

Amphiphilic characteristics can be introduced in starch by treating it with cyclic dicarboxylic acid anhydride, whereas the specific product octenylsuccinate starch (AS) can be synthesized [Caldwell and Wurzburg, 1953; Viswanathan, 1999]. Starch modified with 3 % octenylsuccinic anhydride with a degree of substitution (ds) of 0.015 is a commercially available product. The advantage of octenylsuccinate starch at that ds level is that hydrophilicity is retained, while hydrophobicity is obtained [Viswanathan, 1999]. Increasing the ds does not necessarily increase emulsifying properties, quite the contrary, which was suggested to be caused by octenyl chains tending to fold theirself inside toward the center of the granule, thereby decreasing the interactions between the oil phase and the hydrophobic chains [Viswanathan, 1999]. After synthesis an AS dispersion is treated with acids and/or enzymes to reduce the molecular weight by controlling the viscosity. Better emulsion formation is favoured by higher AS concentrations and/or a decrease in molecular weight during modification [Viswanathan 1999]. Depending on synthesis conditions, e.g. extent of starch hydrolysis, differently manufactured AS types may vary in viscosity and osmotic activity. Both characteristics may provide indications about the polymer's molecular weight and its molecular weight distribution.

The aim of the present preliminary study was to compare two different AS types of unknown molecular weights and degree of substitution with respect to their emulsifying properties. Molecular weight distributions of the tested AS types were compared by means of viscosity and vapour pressure measurements. The influence of the polymer's molecular weight distribution, pH adjustment and drug incorporation, using sodium diclofenac as a model drug, on emulsion properties, i.e. droplet sizes and short term stability, was investigated.

2.2 Experimental section

2.2.1 Materials

Sodium diclofenac (DfNa), purchased from Synopharm (Barsbüttel, Germany), medium chain triglycerides (MCT 812) from Hüls (Witten/Ruhr, Germany), purified castor oil from Henry Lamotte (Bremen, Germany), sorbitol from Caesar & Loretz (Hilden, Germany); double-distilled water was used for all preparations; all used substances were of pharmacopoeial or reagent grade. AS 100 and AS 300, both emulsifying starches, were supplied by National Starch & Chemical (Manchester, United Kingdom) and by Roquette frères (Lestrem, France), respectively.

2.2.2 Experimental methods

2.2.2.1 Flow measurements

Rheological properties were assessed using a rheometer CVO (Bohlin Instruments, Mühlacker, Germany) equipped with a concentric cylinder measuring geometry C25. Viscosity data were derived from the linear region of the flow profile (shear rate 50–300 1/s, n = 50). AS dispersions were prepared (10, 15, 20, 25, 30, 35 and 40 % (w/w)) by dissolving AS in double-distilled water. Four o/w-emulsions containing 10 % (w/w) MCT-castor oil 1:1 stabilized with AS 10 and 15 % (w/w), respectively, were also characterized. Each formulation was measured at 20 °C in triplicate.

2.2.2.2 Osmolality

In order to compare both AS types, the osmotic activities of AS dispersions of different concentrations (10, 15, 20, 25 % (w/w)) were measured. Osmotic activities of investigated preparations were analysed by vapour pressure measurements at 37 °C (vapour pressure osmometer type: No 11.00, Knauer KG, Berlin, Germany; the

apparatus was calibrated with sorbitol solutions within the concentration range of 3.0–8.0 % (w/w); the correlation coefficient obtained was > 0.999).

2.2.2.3 Surface tension

Surface tension measurements were carried out with a thermostatically-controlled Processor Tensiometer K100 (Krüss GmbH, Hamburg, Germany) provided with a Du Noüy ring (ring radius: 9.545 mm, wire diameter: 0.37 mm) at 20 °C. The apparatus was calibrated with double-distilled water achieving a surface tension of 72.14 mN/m. The reduction of surface tension caused by AS 100/300 15 % (w/w), both adjusted to pH 6.5 with 0.1 N-NaOH, was measured in triplicate.

2.2.2.4 Preparation and characterization of AS emulsions

Oil-in-water (o/w) emulsions (10 % oily phase) stabilized with 15 % (w/w) AS 100 or 300 were investigated. The oily phase consisted of MCT 812 and castor oil (1:1). AS was mixed with cold water by stirring gently on a magnetic stirrer until fully dissolved. If necessary, the aqueous dispersion was isotonized with sorbitol and added to the oily phase. A pre-emulsion was prepared using an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) before the emulsion was passed through a high pressure homogenizer (Niro Soavi, type: Panda, Parma, Italy) at room temperature at a pressure of 300 bar (8 cycles, 20 °C) to obtain a submicron emulsion. The pH of the emulsions was adjusted to 6.5 and 7.4 using a 0.1 N sodium hydroxide solution. More details and information on the chosen concentrations and homogenization conditions are given in chapter 4.

In case of drug loaded emulsions the required DfNa amount was dissolved in water and added to a slightly concentrated AS dispersion after pH adjustment. Water was then added to set the necessary AS concentration and the pH was controlled prior to high pressure homogenization.

Particle size distributions depending on the number of homogenization cycles, pH value and DfNa incorporation were determined by laser diffraction (Mastersizer MS

20, Malvern, Worcs, United Kingdom) and calculated by Malvern SB 09 software using the Mie approximation.

2.3 Results and discussion

2.3.1 Rheological properties

Both AS types produce dispersions and emulsions revealing Newtonian flow behaviour, which can be drawn from a linear dependence (R=0.999–1.000) between applied shear rate and shear stress (Figs. 2-1 and -3).

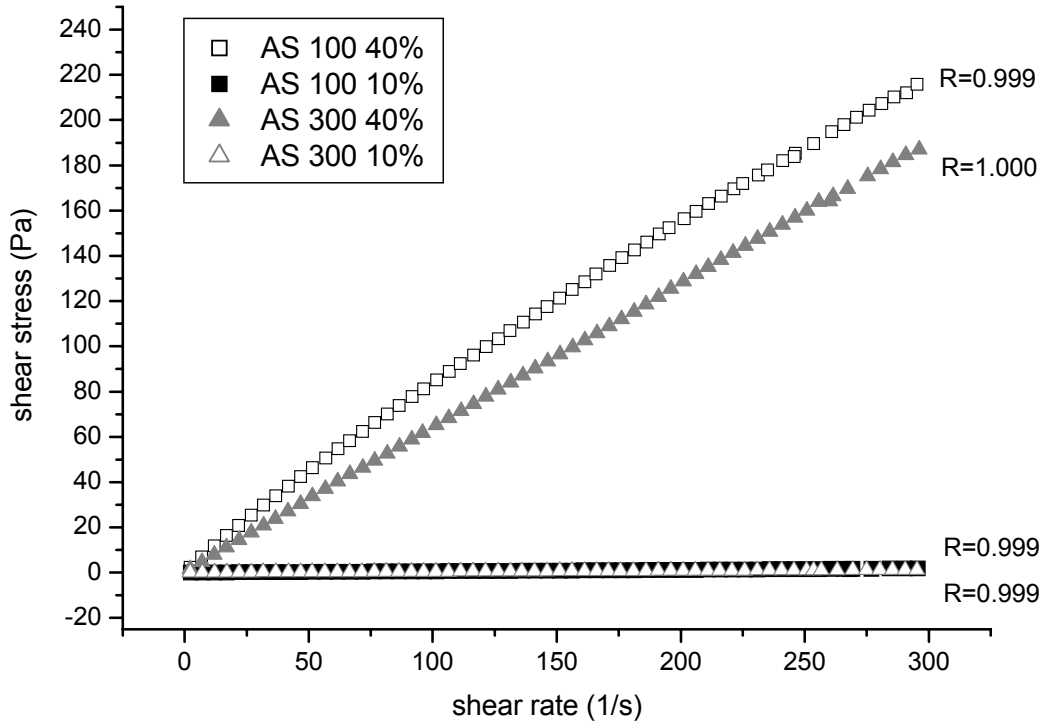

Fig 2-1: Flow profiles of AS 100 and AS 300 dispersions (both 40 and 10 % (w/w)) characterized by a linear increase of shear stress with shear rate (R = 0.999 – 1.000), profiles of AS 100/300 10 % (w/w) overlap.

Fig. 2-2 displays for both AS types tested an increase in viscosity with higher concentrations, whereas AS 100 yields higher viscosities than AS 300, which is an indication of the presence of rather long chain starch molecules. According to the Stokes law, which suggests that the rate of creaming or sedimentation is inversely

proportional to the viscosity, this means that at equal concentrations AS 100 may yield more stable emulsions as compared to AS 300.

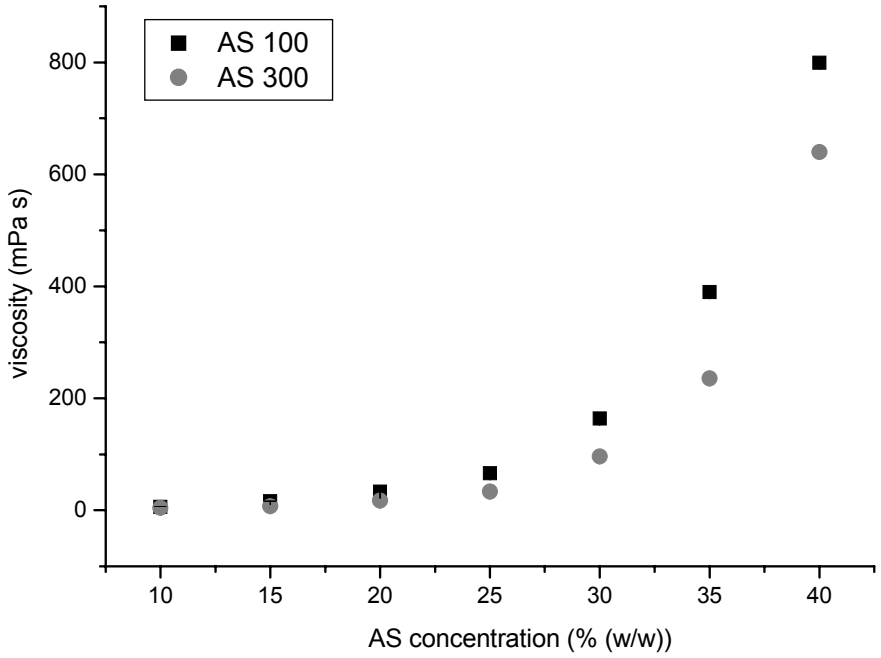

Fig. 2-2: Viscosities of different AS type dispersions depending on concentration (n = 3), mean ± SD, symbol includes standard deviation

Tab. 2-1: Viscosities of different AS type emulsions depending on concentration (n = 3), mean (± SD); emulsions consist of AS/MCT-castor oil 1:1 concentration outer phase/concentration inner phase in % (w/w)

AS 100 15/10	AS 300 15/10	AS 100 10/10	AS 300 10/10
7.1 (± 0.2) mPa s	5.9 (± 0.2) mPa s	39.7 (± 2.6) mPa s	21.2 (± 0.2) mPa s

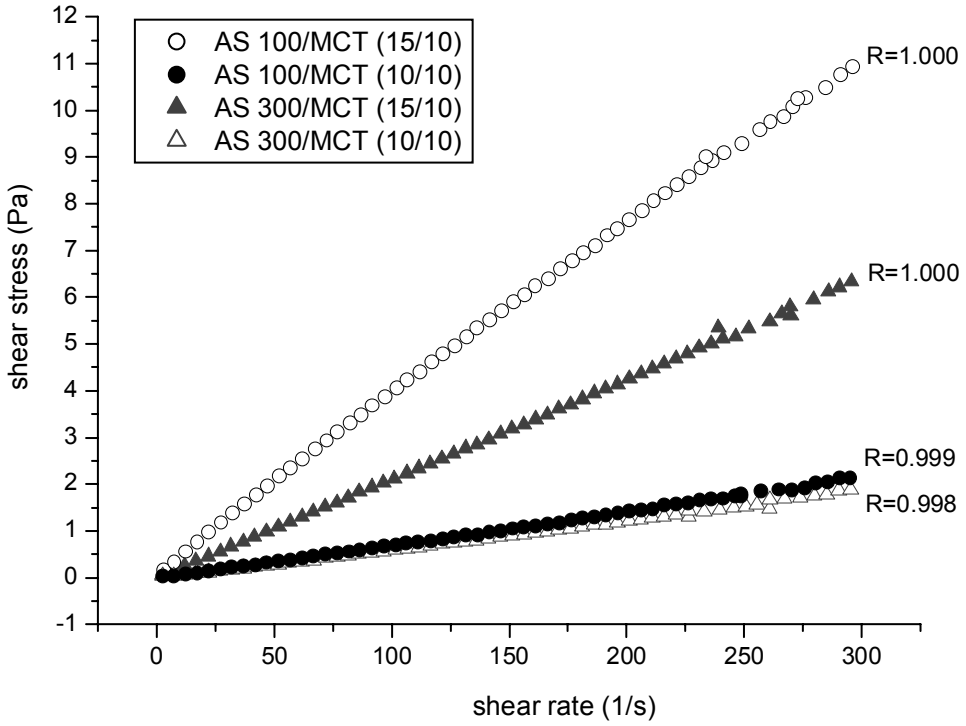

Fig. 2-3: Flow profiles of AS 100 and AS 300 emulsions (both 15 and 10 % (w/w)); inner phase consists of MCT-castor oil 1:1 10 % (w/w); characterized by a linear increase of shear stress with shear rate (R = 0.998 – 1.000).

2.3.2 Osmotic activity

Figure 2-4 shows that, depending on synthesis conditions, e.g. extent of starch hydrolysis, AS vary in their osmotic activities. Higher osmotic activities indicate the presence of short chain starch molecules, which promote a better emulsion formation and finer droplets [Viswanathan, 1999]. AS 100 reveals a stronger osmotic activity than AS 300 which, taking viscosity data into account, indicates a broader molecular weight distribution.

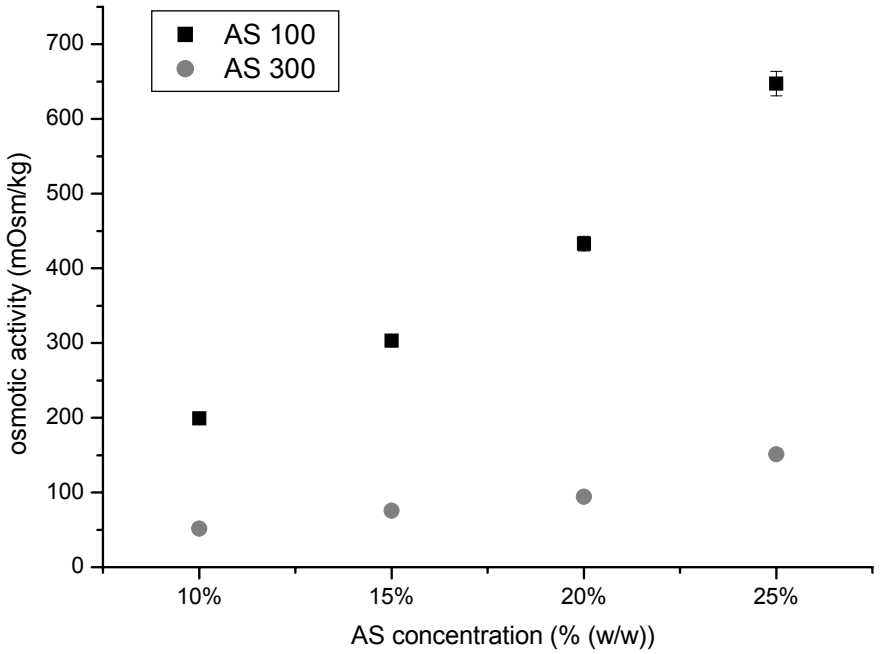

Fig. 2-4: Osmotic activities of different AS type dispersions depending on concentration (n = 3), mean ± SD, symbols include standard deviation

2.3.3 Surface tension

Table 2-2 shows that at pH 6.5 AS 100 lowers surface tension by a greater value, about 34 mN/m than AS 300, which is about 28 mN/m, regardless of the measurement temperature (20 °C or 32 °C). At pH 7.4 the difference seems to be less pronounced.

Tab. 2-2: Decrease of water surface tension (72.14 mN/m) mean (SD) (n=3)

		AS 100 (mN/m)	AS 300 (mN/m)
pH 6.5	20 °C	33.71 (0.62)	28.16 (1.47)
	32 °C	34.39 (0.03)	28.46 (1.58)
pH 7.4	20 °C	30.38 (0.91)	25.49 (1.28)
	32 °C	30.21 (0.35)	26.16 (3.16)

As compared to pH 6.5, both AS types show a slightly diminished decrease of surface tension when the pH value is adjusted to 7.4. As further presented in chapter 4 paragraph 4.3.1.3, the emulsifying character of AS is weakened with pH values higher than 7.

2.3.4 Particle size measurements and emulsion short term stability

Figure 2-5 displays the decrease of the D90 and D50 particle diameters in dependence on the homogenization time. The D90/50 diameter means that 90/50 % of the particles are below the given size. AS 100, under chosen conditions (300 bar, 20 °C), yields emulsions with smaller droplet sizes and decreased particle size distributions already within shorter homogenization times (Fig. 2-5).

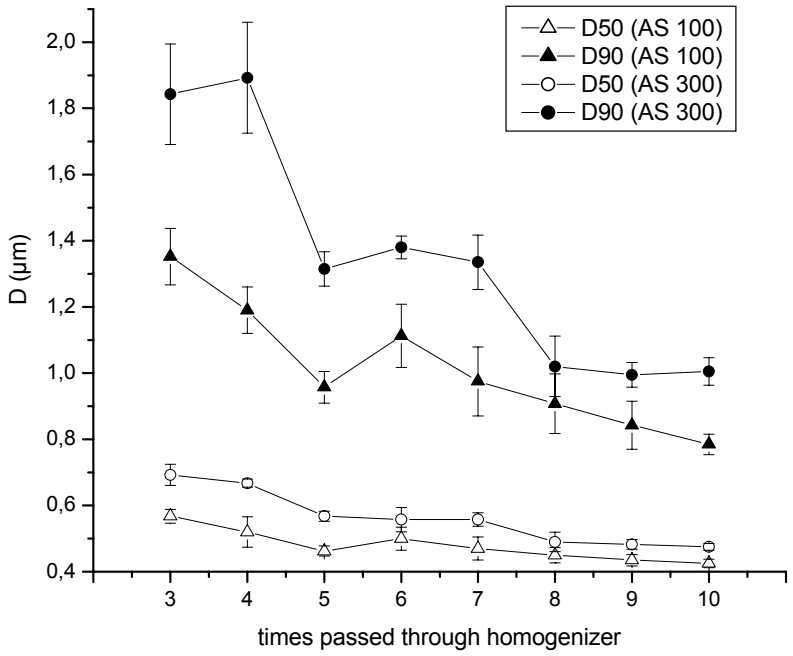

Fig. 2-5: Droplet sizes of o/w emulsions containing AS (15 % (w/w)) and 10 % (w/w) oily phase vs. homogenization time (300 bar, 20 °C)**,** mean ± SD (n = 9)

This is in accordance with osmolarity data which suggested that AS 100 enhances emulsion formation as a result of the presence of short chain molecules.

As AS loses its emulsifying character when the pH is adjusted to 7 or higher (see also chapter 4, paragraph 4.3.1.3) the emulsions presented in the tables 2-3 and -4 were adjusted to pH 6.5.

Tab. 2-3: Droplet sizes of emulsions (300 bar, 8 cycles) after pH adjustment to pH 6.5 shortly after homogenization and after 24 h; mean (SD) (n = 9)

		after homogenization		after 24 h	
		before pH adjustment	**after pH adjustment**		
AS 100	**D90 (µm)**	0.84 (0.04)	0.84 (0.04)	0.84 (0.04)	0.82 (0.03)
	D50 (µm)	0.42 (0.02)	0.43 (0.02)	0.42 (0.3)	0.44 (0.02)
AS 300	**D90 (µm)**	0.98 (0.06)	0.99 (0.05)	not detectable due to coalescence/creaming	
	D50 (µm)	0.53 (0.02)	0.51 (0.04)		

Tab. 2-4: Droplet sizes of AS emulsions (300 bar, 8 cycles) containing DfNa 0.1 % (w/w) shortly after homogenization and after 24 h (pH adjusted to 6.5); mean (SD), (n = 9)

		after homogenization	after 24 h
AS 100	**D90 (µm)**	0.80 (0.02)	0.81 (0.03)
	D50 (µm)	0.45 (0.02)	0.45 (0.02)
AS 300	**D90 (µm)**	1.03 (0.05)	not detectable due to coalescence/creaming
	D50 (µm)	0.49 (0.04)	

In the case of AS 100 stabilized systems, short term stability is not disturbed by pH adjustment (pH 6.5) or drug incorporation. By contrast, all tested AS 300 emulsions tend to destabilize within 24 h. As this can be observed for all AS 300 emulsions, it can be concluded that destabilization is not directly caused by drug incorporation or pH adjustment but by the weaker stabilizing character of AS 300 itself. Under the preparation conditions given, AS 100 is a more effective stabilizer.

2.4 Conclusion

In case of unknown molecular weights, molecular weight distribution and substitution degree the determination of viscosity and osmotic activity of AS dispersions may provide useful information about emulsifying properties. The AS type that exhibits a larger molecular weight distribution, i. e. AS 100, may contribute more effectively to the formation of fine and stable emulsions, which are less susceptible to drug incorporation or pH variations. Emulsions composed with AS 300 tend to destabilize more quickly.

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3 Stability and compatibility/interaction studies on sodium diclofenac using microcalorimetry and differential scanning calorimetry

3.1 Introduction

Diclofenac (Df), a non-steroidal anti-inflammatory drug (NSAID), is a widely spread active substance in solid dosage forms [Al Gohary, 1998]. In eye drop formulations it is used as an alternative to topical steroids to avoid postoperative eye inflammation [Agata et al., 1984; González-Peñas et al., 1998]. Due to is low solubility it is commercially available as sodium diclofenac (DfNa) [Palomo et al, 1999].

Recently it was shown that *n*-octenylsuccinate starch (AS), an emulsifying water soluble nutrition supplement, can improve the in vitro corneal permeation activity of DfNa [Baydoun and Müller-Goymann, 2003].

Information about interaction potential are crucial in preformulation studies with new excipients since certain drug-excipient interactions can change stability and bioavailability of a product [Al Gohary, 1998]. In order to assess information on compatibility and stability of drugs in combination with excipients different methods, like differential scanning calorimetry (DSC) [Al Gohary, 1998; Palomo et al, 1999] and isothermal heat conduction calorimetry, using a thermal activity monitor (TAM), can be applied [Selzer et al., 1998]. Although thermal analyses do not replace chemical methods, valuable information about degree of interaction and change in characteristic features of a drug can be obtained.

The literature describes different instabilities that can be observed during Df storage clearly depending on storage conditions. While the cyclization of Df to an indolinone derivative could only be detected in Df tablets that were stored under severe conditions (90 °C, 55% relative humidity (rH)) for 20 days but not in samples kept at 40 °C, 50% rH for 28 days or in commercial tablets [Eyjolfsson, 2000]. The formation of two oxidative degradates found in solution and the solid state has also been described [Eyjolfsson, 2000]. On the other hand it could be shown that the formation of an inclusion complex with *β*-cyclodextrin improved thermal stability of DfNa in the solid state [Ćwiertnia et al., 1999].

To measure the extent of interaction between DfNa and AS, microcalorimetry measurements were carried out. In order to avoid signals caused by chemical decomposition of DfNa, moderate temperatures (20 and 40 ºC) at high humidity (100% rH) were chosen for storage and measurements. For further characterisations of treated and untreated DfNa DSC and HPLC measurements were performed [Palomo et al, 1999].

3.2 Experimental section

3.2.1 Materials

DfNa was purchased from Caesar & Loretz (Hilden, Germany), AS type 100, an emulsifying starch, was supplied by National Starch & Chemical (Manchester, United Kingdom);
Acetonitrile and acetic acid (both HPLC grade) were obtained from J.T. Baker (Deventer, the Netherlands). Double-distilled water was used for all preparations.

3.2.2 Experimental methods

3.2.2.1 Isothermal heat conduction microcalorimetry (IHCC)

The calorimeter used for these studies was the 2277 Thermal Activity Monitor (TAM, Thermometric AB, Jarfalla, Sweden). Two calorimeter units, which were kept in 25-l water bath to maintain a temperature constance of $< \pm 2 * 10^{-4}$ K, were installed and run simultaneously. Each unit consists of a measuring and a reference channel. Difference in heat flow (P in µW) and heat output (Q in J) between sample and reference were recorded as a function of time. Powder samples were placed in a tightly sealed 3-ml glass ampoule with a teflon-coated butyl rubber disc and an aluminium cap. The amounts weighted were 200 mg and 400 mg for the mixtures in compatibility studies. Before lowering the ampoules to the measuring position they were equilibrated for at least 20 min to achieve the corresponding temperature. After that the sample vessel and a blank reference vessel were slowly lowered into the measurement position. Heat flow signals were monitored by the Digitam 4.0 software (Thermometric AB, Jarfalla,

Sweden). The apparatus was calibrated using the electrical calibration system between 30 and 300 µW, depending on the heat flow expected. Calibration was carried out when the measuring range was changed or the apparatus was switched off. Measurements were performed at 20 and 40 °C. Relative humidity of 100% was adjusted by inserting a glass vial filled with double-distilled water.

Compatibility studies were analysed using the Digitam 4.0 software program.

3.2.2.2 HPLC conditions

The HPLC system from Waters (USA-Milford MA) consisted of a 486 tuneable absorbance detector, a 712 plus autosampler and two 515 HPLC pumps. Separation was achieved with an analytical Hypersil® ODS (particle size 5 µm) column (125 mm x 4 mm) from Grom (Herrenberg, Germany). The analytical Software Millenium 32 from Waters (Milford, MA, USA) was used to determine the peak sizes of the amounts of DfNa.

The mobile phase consisted of double-distilled water/acetonitrile/acetic acid (50/50/2 and 60/40/0.1). The components' amounts were varied to achieve different polarities and thus retention times (R_t) for DfNa in order to detect side peaks (50/50/2, R_t≈3 min, 60/40/0.1, R_t≈10 min). The flow rate was 1.6 ml/min and DfNa was monitored spectrophotometrically at 276 nm. The experiments were run for at least one hour.
Linear correlation between peak area and DfNa concentrations was obtained within the concentration range of 0.05–25 µg/ml. The correlation coefficient was 0.999.

3.2.2.3 Differential scanning calorimetry (DSC)

DSC thermograms were recorded with a Thermal Analysis System SSC 5200, Software: MAS 5700 MA-Station Version 3.2, SSC 5200H Disk-Station Version 3.2, Version/Typ: DSC 220C, Seiko Instruments, Tokyo, Japan). The apparatus was calibrated with indium and tin which were provided by the manufacturer. Samples weighing between 4 – 8 mg were placed in vented aluminium samples pans. Scanning runs were performed under atmospheric conditions within the range of 50–320 °C at a heating rate of 6 K min^{-1}.

Moisture contents were determined by thermogravimetry (TG). Samples of 3–6 mg were weighed directly into open aluminium samples pans. Thermal analyses were conducted at atmospheric conditions. Scans were carried out from 20–120 °C (kept at 120 °C for 10 min under dynamic air flow) at a heating rate of at 5 K min^{-1}. The equipment was calibrated with indium and tin. The loss of mass was recorded and analysed using software mentioned above.

3.3 Results and discussion

3.3.1 HPLC

Within the storage period of 8 d none of the HPLC chromatograms (storage conditions: 20/40 °C at a relative humidity of 100%) showed additional peaks, indicating decomposition of DfNa. DfNa contents did not change within 8 d storage time. This means that signals, which occur in calorimetry studies within 8 d, where DfNa is kept under the same conditions, are not due to decomposition.

3.3.2 DSC and TG

The water amounts taken up by DfNa at 20 °C, determined by TG (Fig. 3-1), in dependence on time first show a linear increase approaching a limit value of 18.84 %. This is in accordance with the literature [Fini et al., 2001] which presented a value of 18.38 % corresponding to four water molecules per molecule DfNa. Plotting

the heat flow volume, taken from the heat flow calorimetry profile, versus storage time results in a similar curve shape (Fig. 3-2).

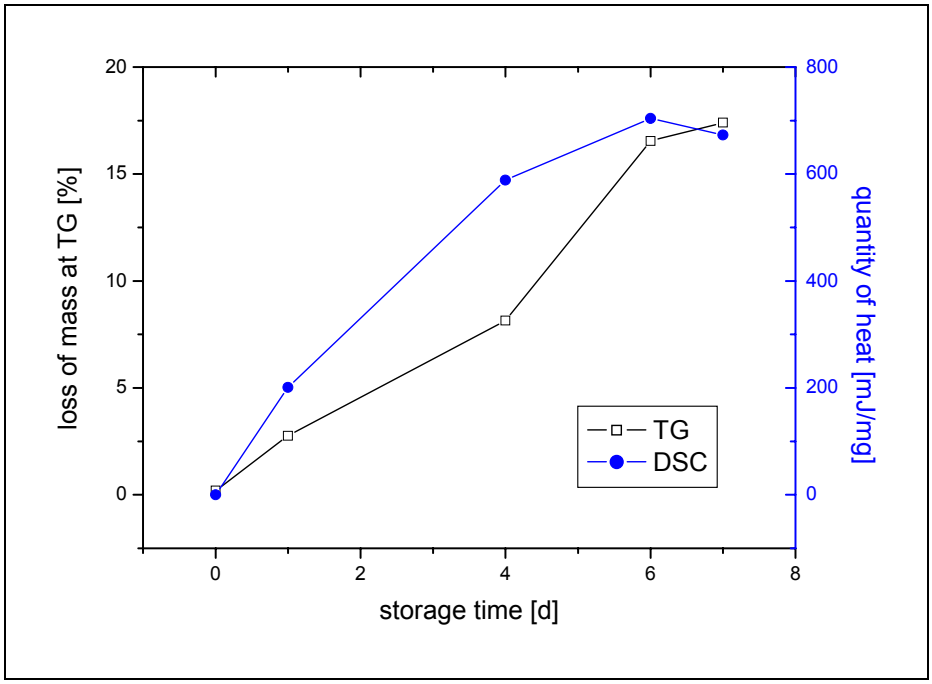

Fig. 3-1: Water amounts taken up by DfNa at 20 °C, determined by TG (squares), and heat quantity measured by DSC (circles) versus storage time

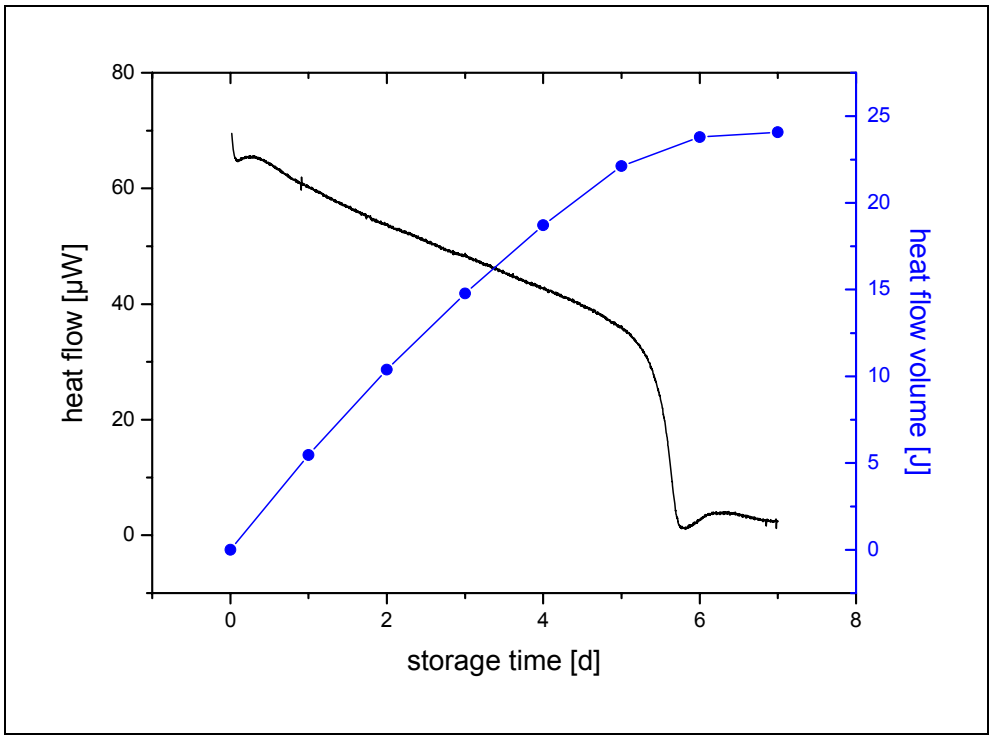

Fig. 3-2: Heat flow conduction calorimetry profile of DfNa at 20 °C/ 100 % rH versus storage time and corresponding heat flow volume curve (circles)

As already described in the literature [Palomo et al, 1999; Fini et al., 2001] DSC curves of untreated DfNa (Fig. 3-3) show an exothermic decomposition signal (278 °C) followed by an endothermic melting signal (281 °C) [Palomo et al, 1999]. As soon as water is taken up by DfNa an additional water evaporation peak occurs starting at room temperature. The longer DfNa has been exposed to moisture the broader gets the signal (Fig. 3-3). Both DSC and TG data point out the formation of a tetrahydrate as already reported [Fini et al., 2001].

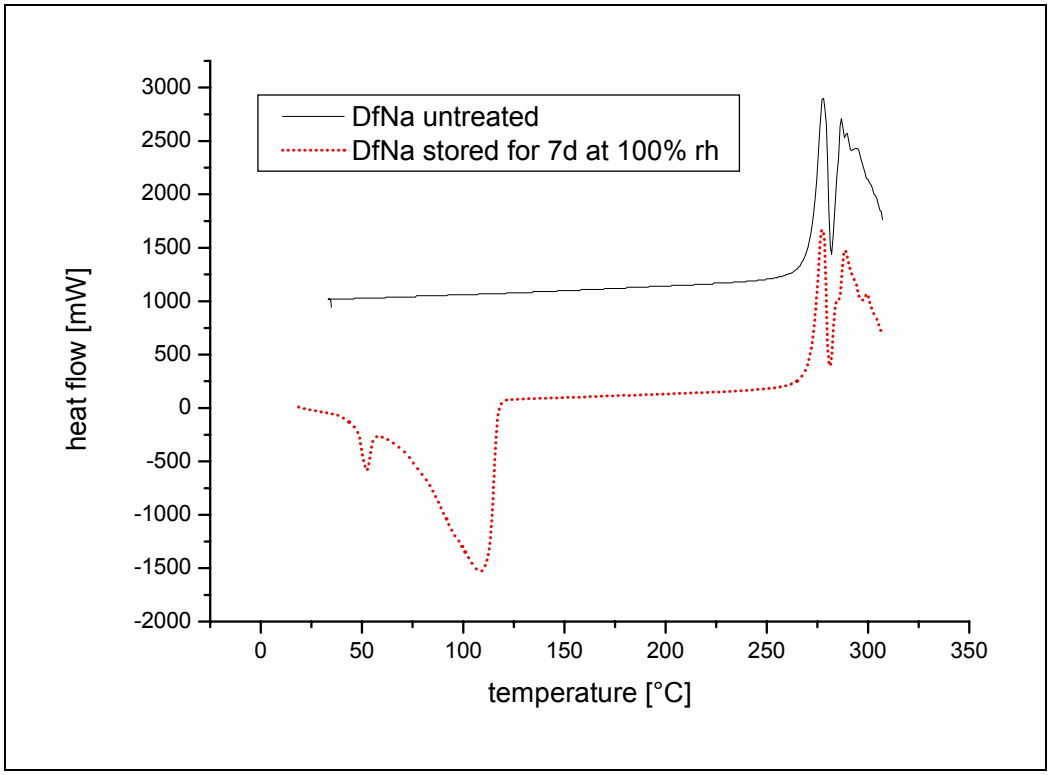

Fig. 3-3: DSC curves of untreated DfNa and DfNa after storage (interrupted line) at 20 ºC/ 100% rH, 7 d

3.3.3 IHCC

Measuring DfNa without defined rH provides a profile close to baseline (Fig. 3-4). Profiles achieved at an rH of 100% reveal first a slight decrease in heatflow and later a distinct peak. Heatflow subsequently approaches baseline. As compared to 40 °C, the profile obtained at 20 °C starts at lower values (86 µW) and drops off more slowly. The peak, which is less pronounced, is notably delayed and occurs after

121 h (Fig. 3-4). Water condensation, water adsorption, formation of hydrates as well as dissolving processes and hydration may cause occurrence of heat flow.

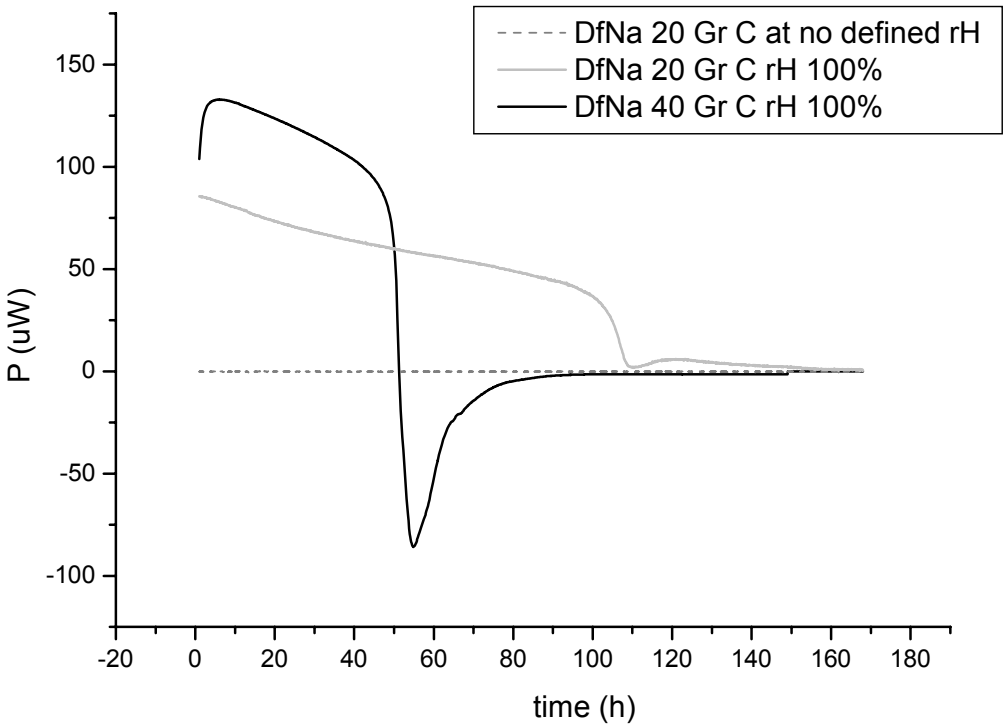

Fig. 3-4: Heat flow profiles of DfNa at 20 ˚C at no defined rH (interrupted line) and at an rH of 100% (grey line) and at 40 ˚C at an rH of 100% (black line)

Fig. 3-5 shows the heatflow measured curves obtained with DfNa, AS 100 and two DfNa-AS 100 1:1-mixtures (mASDfa and –b) using heat flow conduction calorimetry at 20 °C at an rH of 100%. The curve illustrating the difference in heatflow between a calculated/theoretical (thASDf) and the measured curves is rather low indicating weak interaction between AS 100 and DfNa, which indicates an acceptable compatibility/a low level of interaction.

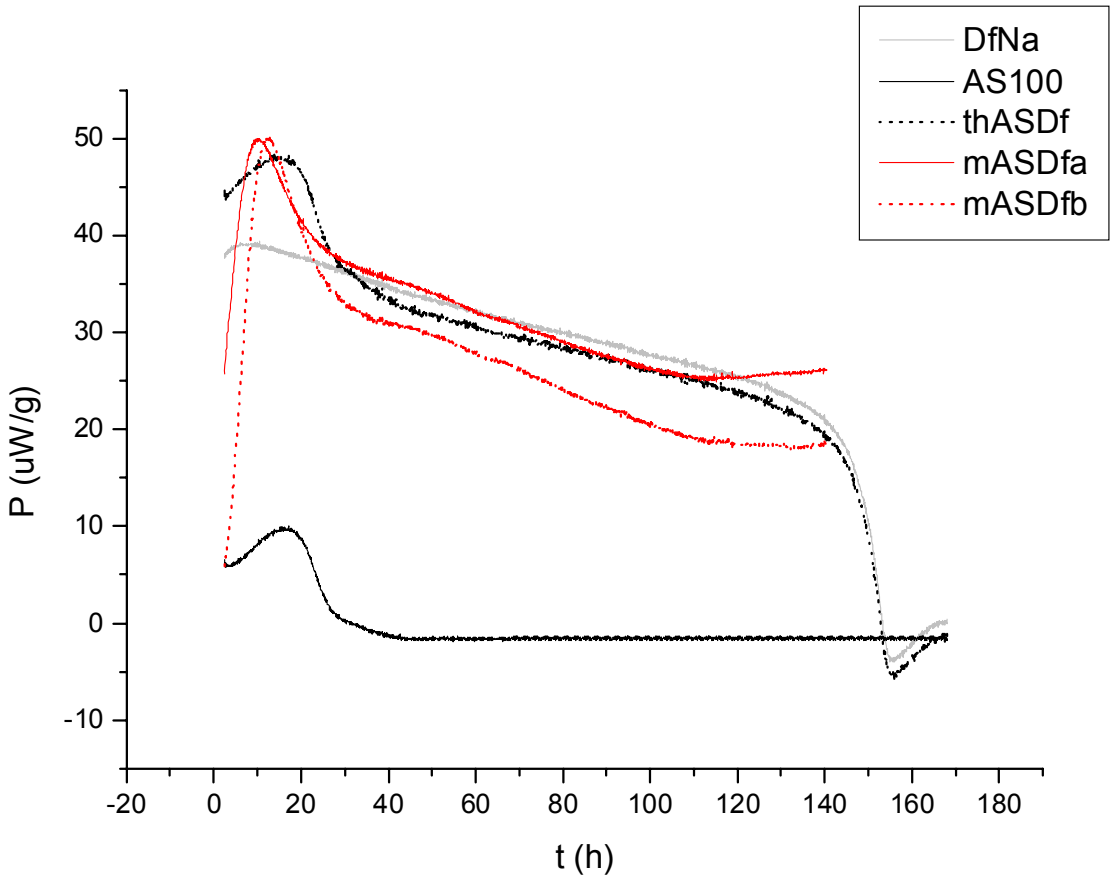

Fig. 3-5: Heat flow conduction calorimetry profile of DfNa and AS 100 at 20 °C/ 100 % rH compared to a mixture DfNa and AS 100 (1:1), mASDf = measured curve (mixture), thASDf = theoretical/calculated curve

3.4 Conclusion

Microcalorimetric profiles indicated low interaction between drug and excipient, while heat signals achieved with DfNa alone are based on a mere physical uptake of water to form a DfNa tetrahydrate and not on chemical decomposition.

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4 Development of *n*-octenylsuccinate starch stabilized emulsions for the non-enteral application route: short and long-term stability studies

4.1 Introduction

Lipophilic drugs are poorly soluble in water and therefore show low dissolution rates which often affects oral bioavailability. Micronization improves the drugs' dissolution rates due to a specific larger surface area [Reverchon et al., 2004]. The incorporation of the lipophilic drug into solid lipid nanoparticles [Hu et al., 2004] or into an oil-in-water (o/w) emulsion [Hansen et al., 2005] may show an increased oral bioavailability.

Accordingly, highly lipophilic drugs can be incorporated in o/w emulsions intended for ophthalmic application to increase the drug's bioavailability. Incorporated emulsifiers must reveal a low irritation potential. Emulsions stabilized with *n*-octenylsuccinate starch (AS) reveal a good eye tolerance [Baydoun et al., 2004].

Since emulsions undergo a number of instability processes, including creaming, flocculation and coalescence, characterization and control of these processes are key aspects of the formulation of commercial products. Spray drying of o/w emulsions is an interesting method to achieve physical, chemical and microbial stabilization of both food and medical emulsions [Christensen et al., 2001; Dollo et al., 2003; Hansen et al., 2005].

Likewise, in the case of ophthalmic and parenteral emulsions it is advantageous to achieve emulsions, which remain stable during autoclaving. In order to increase the resistance to the thermic shock induced by the autoclaving sterilization co-emulsifiers can be added [Jumaa et al., 1998; Buszello et al., 2000]. A method to obtain useful information on drug/excipient stability, excipient compatibility and the degree of interaction between different chemicals, such as emulsifiers, is the isothermal heat conduction calorimetry (IHCC), using a thermal activity monitor (TAM) [Selzer et al., 1998; Phipps and Mackin, 2000]. Isothermal microcalorimetry is a technique by which the heat flow produced by a chemical, physical or biological process is continuously

monitored while the sample is kept at isothermal conditions [Phipps and Mackin, 2000].

The effort of this investigation was to develop an AS stabilized emulsion formulation intended primarily for ocular application. The amounts of the emulsifying agent AS (5, 10, 15, 25 % (w/w)) and the oil phase (medium chain triglycerides (MCT), MCT-castor oil 1:1, 5, 10, 15 % (w/w)) have been varied. Different co-emulsifiers, which have approved for parenteral and/or ocular application, i. e. an ABA block-copolymer (F68), polyoxyethylene castor oil (POC) and phospholipon 90 G (P90G) [Jumaa and Müller, 1998], have been combined with AS adjusting different concentration combinations in binary mixtures. Additionally the influence of the pH value (pH 4, 6, 6.5, 7 and 7.5) on the emulsifying qualities of AS has been tested. A redispersable dry powder emulsion, stabilized with AS, was prepared by spray drying. Release/liberation experiments were performed with spray dried emulsions and compared with untreated systems.

Short and long-term stability of AS emulsions as well as stability during the autoclaving process have been investigated visually and by laser diffraction. Interactions between emulsifiers were examined visually and were confirmed by IHCC.

4.2 Experimental section

4.2.1 Materials

DfNa was purchased from Synopharm (Barsbüttel, Germany) and Caesar & Loretz (Hilden, Germany), medium chain triglycerides (MCT 812) from Hüls (Witten/Ruhr, Germany), purified castor oil from Henry Lamotte (Bremen, Germany), hydroxypropylmethylcellulose (HPMC) type Metolose 90 SH 400, substitution type 2208, USP from Shin Etsu (Tokyo, Japan), sorbitol from Caesar & Loretz (Hilden, Germany), thimerosal from Synopharm (Barsbüttel, Germany), sodium hydroxide from Merck (Darmstadt, Germany); Pluronic® F68 (F68) and Cremophor® EL (POC) (both BASF, Ludwigshafen, Germany), Phospholipon® 90 G (P90G) (Nattermann,

Köln, Germany); acetonitrile and acetic acid (both HPLC grade) were obtained from J.T. Baker (Deventer, the Netherlands); sodium chloride, potassium dihydrogen phosphate and disodium hydrogen phosphate (all pro analysi), purchased from Merck (Darmstadt, Germany), were used to prepare isotonic phosphate buffer pH 7.4 (PBS 7.4) according to the German Pharmacopoeia (DAB 2001); all substances used were of analytical or pharmacopoeial grade. Thimerosal 0.004 % (w/w) was added to all preparations for preservation.

AS type 100, an emulsifying starch which is further referred to as AS, was supplied by National Starch & Chemical (Manchester, United Kingdom). Double-distilled water was used for all preparations.

4.2.2 Experimental methods

4.2.2.1 Emulsion preparation

Besides the variation of the homogenization time, figure 4-1 illustrates the modifications tested to investigate emulsion formation and stability. AS 100 stabilized oil-in-water (o/w) emulsions with different oil phases (MCT 812 and MCT 812/castor oil (1:1)) and different AS-oil ratios (5/10/15 and 5/10/15/25 % (w/w)) were prepared. AS was mixed with cold water by stirring it gently on a magnetic stirrer until fully dissolved. AS moisture contents were previously determined by thermogravimetry (Thermal Analysis System SSC 5200, Software: MAS 5700 MA-Station Version 3.2, SSC 5200H Disk-Station Version 3.2, Version/Typ: DSC 220C, Seiko Instruments, Tokyo, Japan).

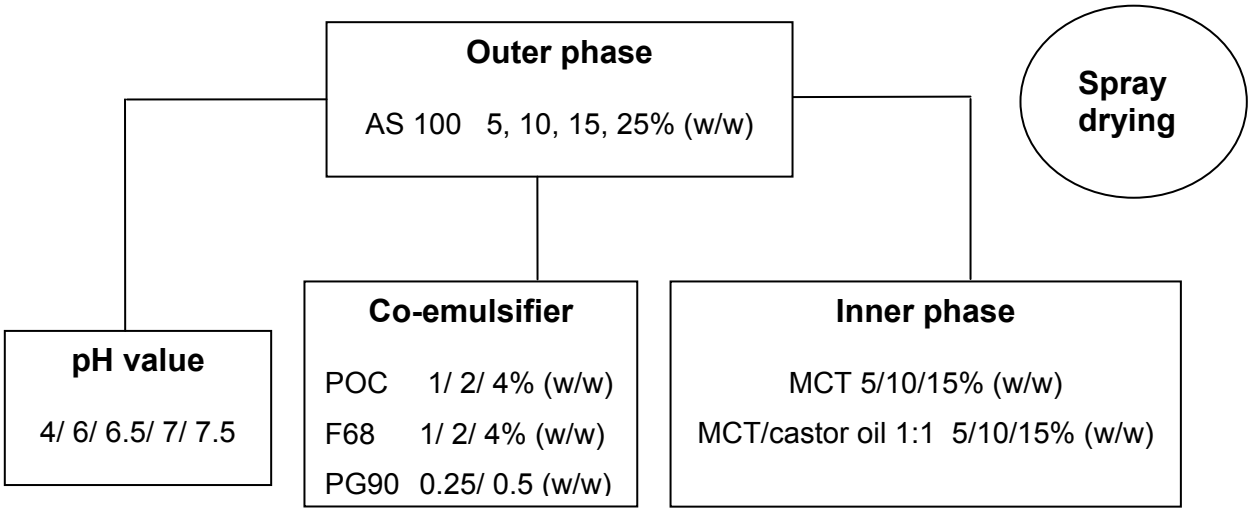

Fig. 4-1: Different variations tested to investigate AS emulsion formation and stability.

The aqueous dispersion was added in a stepwise manner to the oil phase and a pre-emulsion was prepared (1 min, 8000 rpm) using an Ultra-Turrax (Janke & Kunkel, Staufen, Germany). The coarse pre-emulsion was passed at room temperature through a high pressure homogenizer (Niro Soavi, type: Panda, Parma, Italy) to obtain a submicron emulsion. In order to optimize the homogenization process of the specific emulsion, the system was passed repeatedly through the homogenizer monitoring achieved droplet diameters versus the number of homogenization cycles. The required pH was adjusted with a 0.1 N NaOH solution prior to and after emulsion preparation.

Furthermore, to increase and investigate emulsion stability during autoclaving (121 °C, 2 bar, 20 min) three different co-emulsifiers, an ABA block-copolymer, Pluronic F68 (F68), polyoxyethylene castor oil (POC) and phospholipon 90 G (P90G) were combined with AS adjusting different concentration combinations in binary mixtures.

DfNa loaded emulsions were prepared as described in chapter 2, paragraph 2.2.2.4.

To test the influence of the pH value emulsions (AS 10 % (w/w), MCT 10 % (w/w)) were adjusted to 6, 6.5, 7 and 7.5 using a 0.1 N NaOH solution. Osmolality was

adjusted with sorbitol, if necessary, using a calibrated vapour pressure osmometer (Wescor 5500, Baumann-Medical, Wetzikon, Switzerland).

Dry powder emulsions were achieved by spray-drying a stock emulsion (AS 100 25 % (w/w), MCT 15 % (w/w)), pH was adjusted to 6.5 (1 N NaOH), on a Büchi spray dryer (Büchi Labortechnik, Flawil, Switzerland) at 180 ºC inlet temperature and 95 ºC outlet temperature as recommended for AS 100 by the manufacturer (National starch & Chemical). Furthermore an emulsion containing AS 10 % (w/w), and MCT/castor oil (1:1) 10 % (w/w) was spray dried (inlet 180 °C, outlet 95 °C). The moisture content of the dry emulsion was determined by thermogravimetry (see above).

The dry emulsion was reconstituted in double distilled water using a magnetic stirrer.

4.2.2.2 Liberation studies

Liberation profiles for an AS 100 emulsion (7.5 % (w/w) and AS 100 (7.5 % (w/w)) reconstituted spray dried emulsion were compared. The stock emulsion contained 22.5 % (w/w) AS 100, MCT 15 % (w/w) and DfNa 0.3 %, pH was adjusted to 6.5. The stock emulsion was diluted in double-distilled water adjusting 7.5 % AS and 5 % MCT (DfNa 0.1 %). The spray dried (inlet 180 °C, outlet 95 °C) stock emulsion was reconstituted in double-distilled water just the same after determination of water content by thermogravimetry (see above).

An HPMC emulsion 0.75 % (w/w), containing 5 % (w/w) MCT and DfNa 0.1 % (w/v) prepared by high pressure homogenization (400 bar, 10 cycles) was also measured. pH of all systems was adjusted to pH 6.5 with a 0.1 N NaOH solution.

Studies were performed throughout 6 h with a modified Franz diffusion cell [Franz, 1975] as described elsewhere [Refai and Müller-Goymann, 1999]. The donor compartment was filled with the polymer drug solutions whereas isotonic phosphate buffer pH 7.4 (PBS) was used as acceptor medium. Analysis was performed by UV detection at 276 nm.

4.2.2.2 Emulsion characterization

Particle size distribution was analysed by laser diffraction (Mastersizer MS 20, Malvern, Worcs, United Kingdom) and calculated by Malvern SB 09 software using the Mie approximation. The emulsions were further characterized by transmission electron microscopy (TEM) [Friedrich and Müller-Goymann, 2003] and scanning electron microscopy (SEM) [Reichl, 2003] as described in the literature. Emulsions were stored at room temperature and observed for more than 12 months.

4.2.2.3 Emulsifier interaction studies using isothermal heat conduction calorimetry (IHCC)

The calorimeter used for these studies was the 2277 Thermal Activity Monitor (TAM, Thermometric AB, Jarfälla, Sweden). Two calorimeter units, which were kept in the 25-l water bath of the microcalorimeter to maintain a temperature constancy of $< \pm 2 * 10^{-4}$ K, were installed and run simultaneously. Each unit consists of a measuring and a reference channel. Difference in heat flow (P in µW) and heat output (Q in J) between sample and reference were recorded as a function of time. Powder samples were placed in a tightly sealed 3-ml glass ampoule with a teflon-coated butyl rubber disc and an aluminium cap. The amounts weighted were 200 mg and 400 mg for the mixtures in compatibility studies and 100 mg in stability studies of DfNa. Before lowering the ampoules to the measuring position they were equilibrated for at least 20 min to achieve the corresponding temperature. After that the sample vessel and a blank reference vessel were slowly lowered into the measurement position. Heat flow signals were monitored by the Digitam 4.0 software (Thermometric AB, Jarfälla, Sweden). The apparatus was calibrated using the electrical calibration system between 30 and 300 µW, depending on the heat flow expected. Calibration was carried out when the measuring range was changed or the apparatus was switched off. Measurements were performed at 20 and 40 °C. Relative humidity of 100 % was adjusted by inserting a glass vial filled with double-distilled water.

4.3 Results and discussion

4.3.1 Emulsion properties and particle size distribution depending on process parameters, pH value and emulsion composition

4.3.1.1 Influence of the homogenization time

AS stabilized emulsions (Fig. 4-2), containing MCT as inner lipophilic phase (AS and MCT 10 % (w/w) each) prepared by high-pressure homogenization reveal monomodal particle size distributions with droplet sizes around 0.5 µm and a D 90 value below 1 µm already at moderate homogenization conditions (300 bar, 4 cycles, 20 °C).

An AS stabilized emulsion containing AS 100 and MCT, 10 % (w/w) each, was prepared by high pressure homogenization (300 bar, 20 °C). Figure 4-2 shows the particle diameters (D90, D50 and D10) of the emulsion as a function of cycle numbers. In the beginning D90, D50 and D10 values are continuously decreased, i.e. 1.06 µm (D90), 0.50 µm (D50) and 0.24 µm (D10) for cycle 3 and 0.90 (D90), 0.45 µm (D50) and 0.20 µm (D10) for cycle 5. After remaining constant between cycle 5 and 9, there is a clear particle diameter increase, especially of the D90 value, which is an indication of too much energy given to the emulsion. Stabilization with AS 100 yields emulsions with a monomodal particle size distribution (300 bar, 5 cycles, 20 °C) (results not shown).

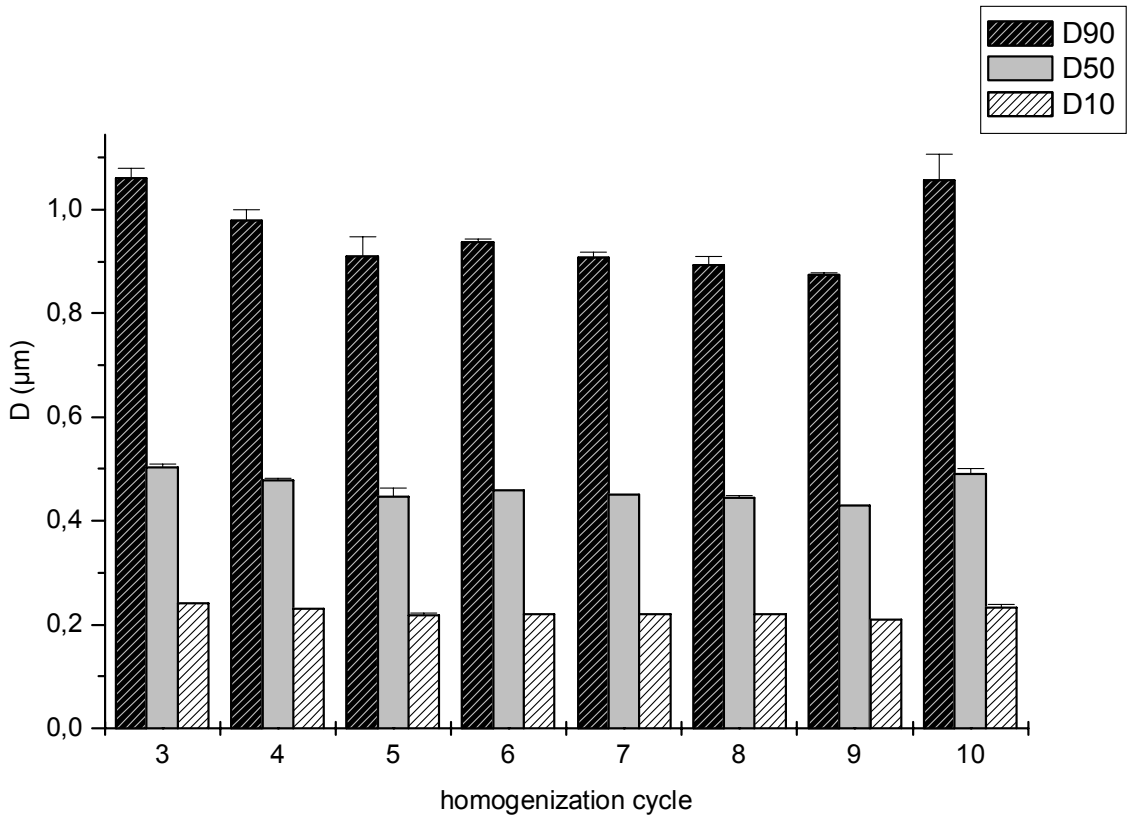

Fig. 4-2: Particle size distribution of an AS-MCT emulsion (10 %/10 % (w/w)) depending on the number of homogenization cycles (300 bar, 20 °C). Mean ± SD (n = 3), D90 (black columns), D50 (grey columns), D10 (white columns) (µm).

4.3.1.2 Influence of the AS/MCT concentration ratio

While AS/MCT ratios of 5:5 and 10:10 yield similar particle sizes, the 15:15 ratio provides emulsions with smaller droplets (Fig. 4-3). Results achieved with AS 5 % (w/w) do not significantly differ when the amount of MCT incorporated is raised from 5 to 15 % (w/w). Raising the AS concentration to 15 % (w/w) generally leads to smaller droplets and even results in a smaller particle size distribution upon homogenization with an equal MCT amount. At this specific AS concentration equal MCT concentrations seem to induce a more effective and more consistent homogenization process possibly in consequence of a higher shear effect.

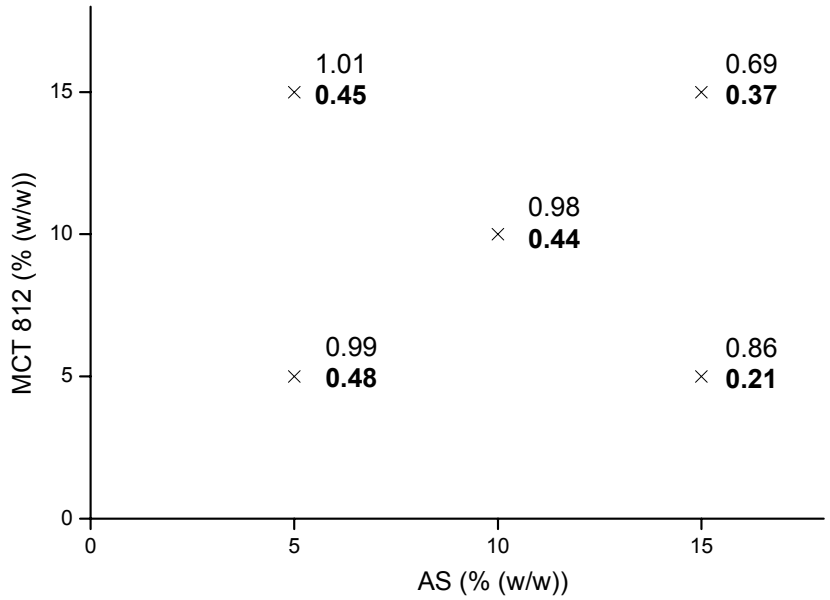

Fig. 4-3: AS-MCT concentration combinations tested D90 and D50 (bold) mean values (µm) (n=3). Emulsions were prepared by high pressure homogenization (300 bar, 20 °C, 4 cycles).

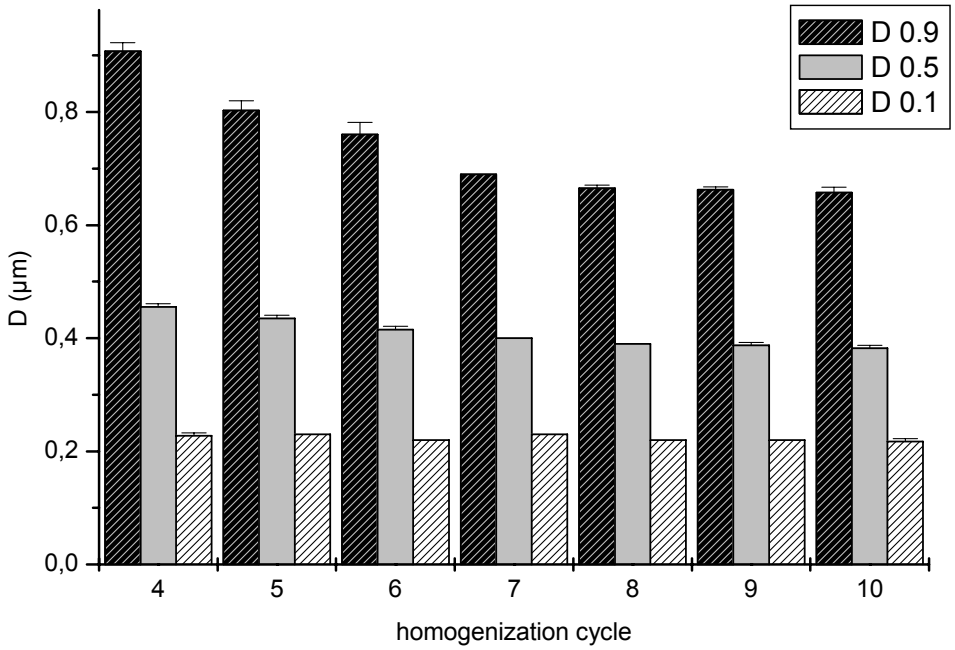

Fig. 4-4: Particle size distribution of an AS-MCT emulsion (25 %/15 % (w/w)) depending on the number of homogenization cycles (450/50 bar, 20 °C). Mean ± SD (n = 3), D90 (black columns), D50 (grey columns), D10 (white columns) (µm)

Furthermore, an emulsion containing AS 100 25 % (w/w) and MCT 15 % was prepared by high-pressure homogenization applying a pressure of 450 bar at the first stage and 50 bar at the second stage (Fig. 4-4). While most of the oil globule reduction takes place in the first stage, there is a tendency for clumping or clustering

of the reduced oil globules. The second stage valve permits the separation of those clusters into individual oil globules. To avoid heating, the emulsion was prepared under cooling with water (18 °C). D50 values of 0.45 µm are reached after the 4th cycle and are decreased to 0.38 µm after the 6th cycle. The D90 values get smaller, from 0.91 µm after cycle 4 and 0.68 after cycle 7. Particle sizes remain constant between cycle 7 and cycle 10.

Figure 4-5 presents a TEM image of an AS stock emulsion (AS 25 % (w/w) and MCT 15 % (w/w)), where the oil droplets (sizes far below 1 µm) seem to be tighly packed. As can be drawn from figure 4-6, these highly concentrated emulsions remain stable over longer than 14 months (20 °C storage temperature). It is well known that concentrated emulsions like mayonnaise do not need stabilizing, since the crowding effect of the droplets is sufficient to prevent creaming. Therefore, it can be beneficial to increase the apparent viscosity of the product (without exceeding a critical concentration) to form a stable continuous network. Stock emulsions can be spray dried or diluted prior to usage.

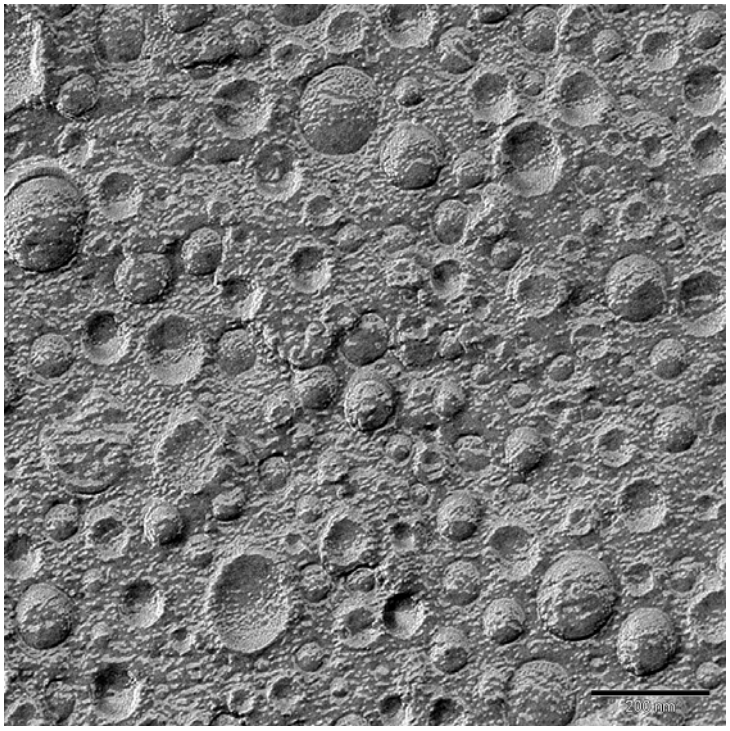

Fig. 4-5: TEM image of an AS stock emulsion containing AS 25 % (w/w) and MCT 15% (w/w).

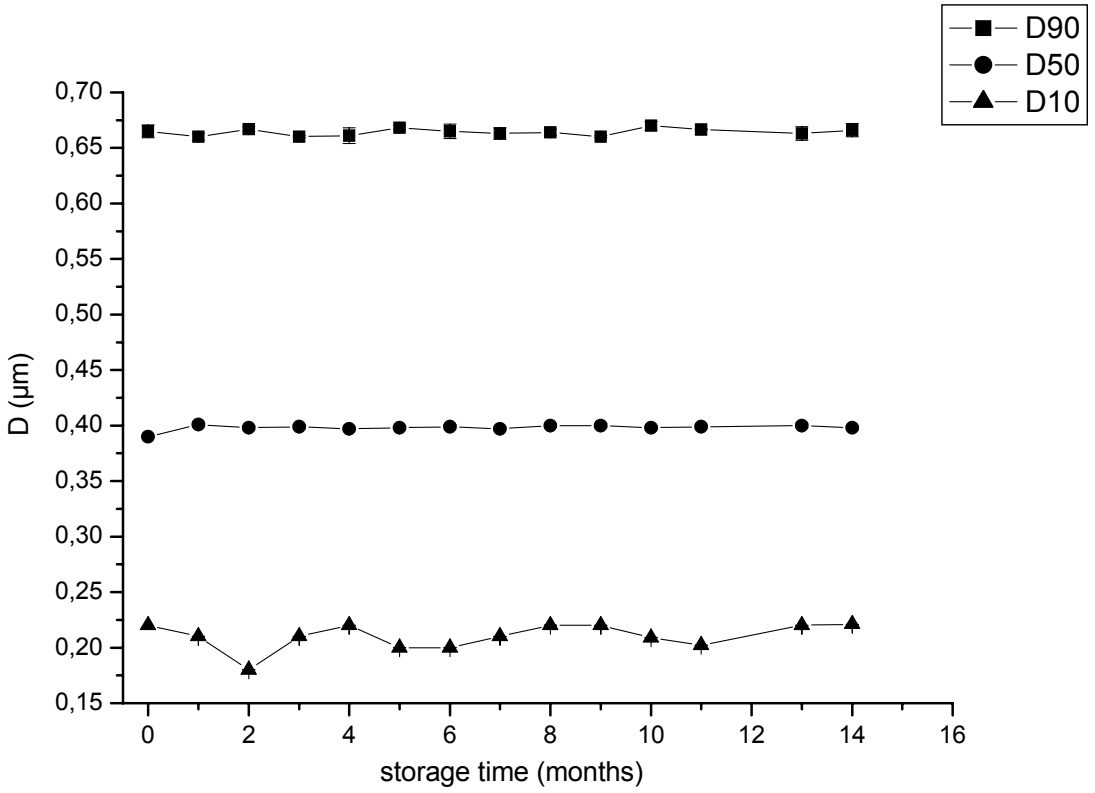

Fig. 4-6: Stability of an AS emulsion (AS 25 %, MCT 15 % (w/w)) at 20 °C; Mean ± SD (n = 3); symbols include standard deviations

As comparing the results obtained in Fig. 4-6 with the droplet sizes in Fig. 4-5 the limits of laser diffraction measurements become obvious since the extremely high presence of particles below 200 nm (Fig. 4-5) could only be detected by TEM.

4.3.1.3 Stability to pH variations

The pH value of an AS 100 dispersion 15% (w/w) is about 4. Emulsifying properties were tested at this pH value and at pH 6, 6.5, 7 and 7.5. To investigate whether the pH has an influence not only on emulsion stability but also on the emusification procedure, adjustment was once set before preparation of the pre-emulsion and once right after high-pressure homogenization.

It was found that at pH-values above 7 AS completely loses its emulsifying properties. Emulsification with AS dispersions adjusted to pH 7 and higher is

impossible and emulsions adjusted after high-pressure homogenization visibly break within minutes.

As long as the pH value is kept at values lower than 7, droplet sizes and stability are comparable for two corresponding emulsions (i. e. same composition, preparation and storage), no matter if the pH is set before or after homogenization (data not shown). At higher pH values deprotonation of the carboxylic acid functional group may lead to a negatively charged octenylsuccinate side chain. This weakens its lipophilic properties, which in turn may result in a weaker amphiphilic character of the AS molecule as a whole.

If the lipophilic phase is able to additionally lower the pH due to the presence of free fatty acids, as in the case of castor oil, it is advantageous to first adjust the pH after high-pressure homogenization.

Taking factors, such as drug stability, solubility and permeation, into consideration, the pH should be adjusted within a physiologically tolerated pH range, which lies between 5.8 and 11.4 for the ocular site of application [Dolder, 1990]. At pH 6.5, which was finally chosen for the preparation of most AS formulations investigated, AS emulsion, stability and ocular tolerance are well combined.

4.3.1.4 Influence of the oil phase composition

Besides pure MCT, a mixture of MCT/castor oil with a 1:1 ratio as the inner lipophilic phase, was investigated for emulsion formation and stability. MCT 812 has a density of 0.94–0.95 g/cm3 20°C which is close to that of water. In accordance with the law of Stokes a low difference between the densities of the dispersed and continuous phase increases emulsion stability. The incorporation of the relatively polar castor oil allows to prepare emulsions with a capacity for a higher drug uptake [Jumaa and Müller, 1998]. The D50 and D10 values achieved with the corresponding systems (i. e. same AS/lipophiic phase concentration ratio, pH value, homogenization and storage conditions), which only differ in the oil phase composition, are comparable. For an AS emulsion containing AS 10 % (w/w) and MCT/castor oil 1:1 10 % (w/w) the

diameters 0.49 µm (D50) and 0.24 µm (D10) were obtained at 300 bar after 8 cycles. In case of the D90 value it takes more than 8 cycles to decrease this value to data around 1 µm (1.01 µm).

MCT leads to smaller D90 values particle sizes within shorter homogenization times (D90 0.8 µm after 4 cycles) in comparison to an MCT/castor oil mixture, which takes 8 cycles until D90 becomes smaller than 1 µm. This may be due to the higher viscosity of castor oil, which leads to broad particle size distributions. Oil phases with higher viscosities need higher homogenizatioin pressures to obtain small particle size distributions. This was according to expectations as MCT shows a lower viscosity than castor oil.

As presented in figure 4-7 emulsions consisting of AS 10 % (w/w) and MCT/castor oil 1:1 10 % (w/w) destabilize within 3 months. While D50 and D10 values remain stable over 14 months D90 values become larger after 3 months which can also be recognized macroscopically by obvious creaming.

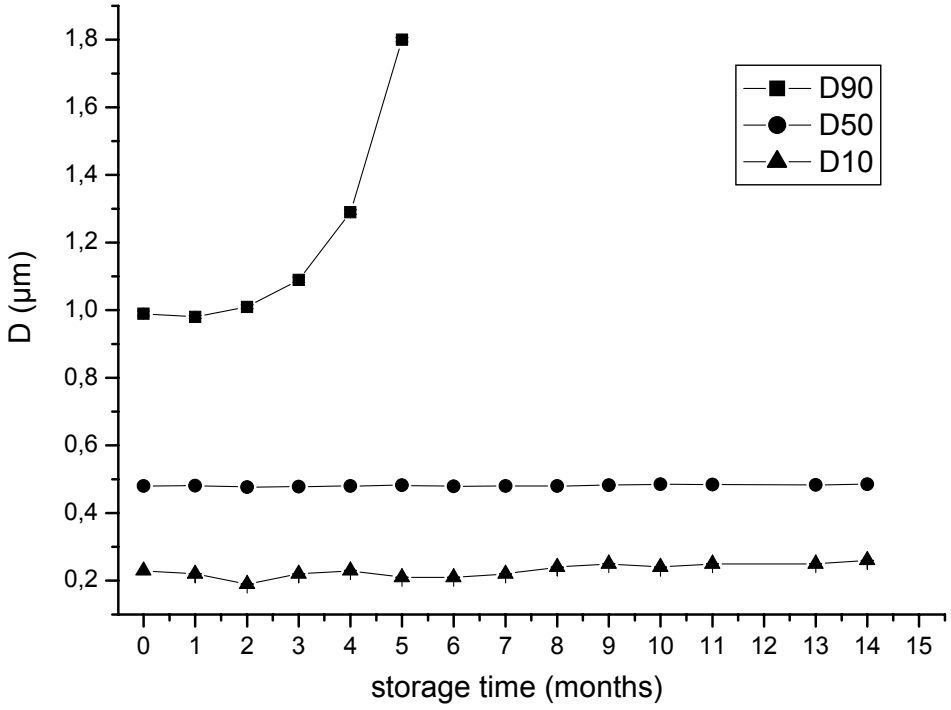

Fig. 4-7: Stability of an AS emulsion (AS 10 %, MCT/castor oil 1:1 10 % (w/w)) at 20 °C; mean ± SD (n = 3); symbols include standard deviations

This problem can be overcome by increasing the emulsifier concentration. Emulsions, which consist of AS 15 % (w/w) and MCT/castor oil (1:1) 10 % (Fig. 4-8) yield emulsions (300 bar, 8 cycles, 20 °C) with (monomodal) particle size distributions comparable to the 10:10 ratio emulsions without castor oil (see results presented in chapter 2, 2.3.4). Stored at room temperature they are stable for at least 6 months.

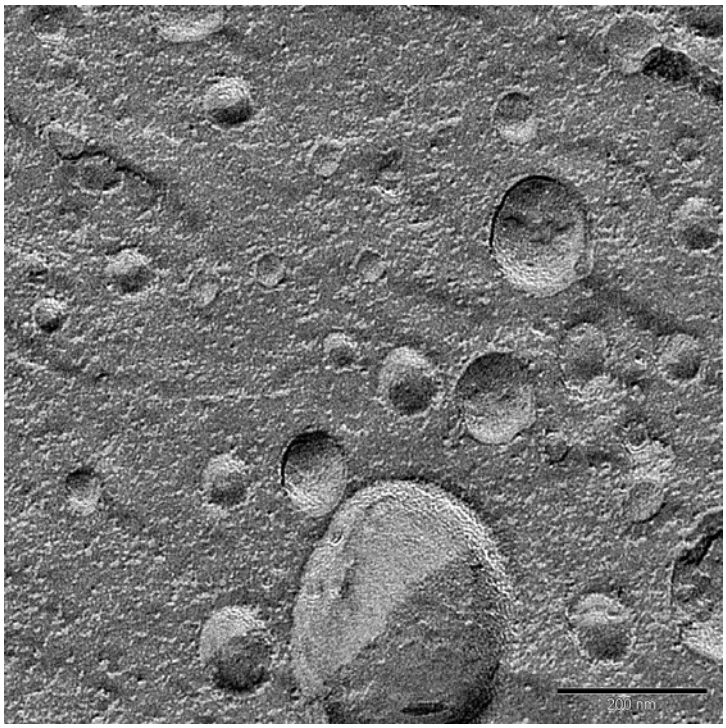

Fig. 4-8: TEM image of an AS emulsion containing AS 15 % (w/w) and MCT/castor oil 1:1 10 % (w/w).

4.3.2 Drug stability/compatibility and emulsifier interaction studies using isothermal heat conduction calorimetry

As AS emulsions of any concentration investigated break throughout autoclaving, different amounts of various co-emulsifiers were added (Fig. 4-1) in order to increase the emulsion stability. Binary mixtures of AS with F68 and POC, respectively, showed complete phase separation already during the pre-emulsification process indicating strong emulsifier interactions. This could also be drawn from the high difference in heat flow achieved in IHCC studies with 1:1 mixtures as compared to a calculated curve from

the heat flows of the pure substances (Figs. 4-9 and -10). The combination with P90G revealed a much weaker interaction level (Fig. 4-11).

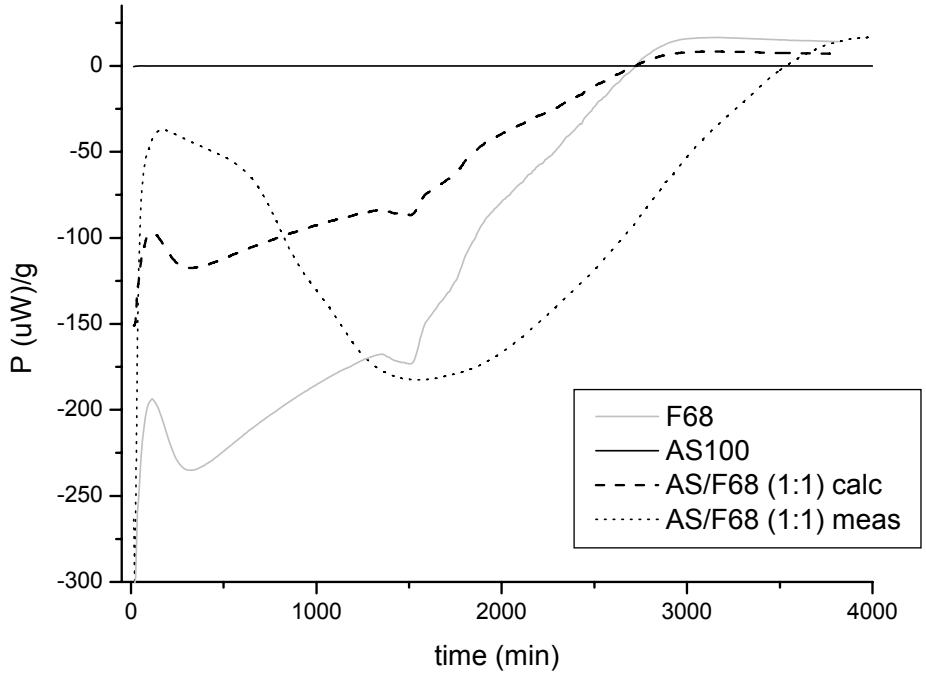

Fig. 4-9: Heat flow profiles at defined rH (100 %) at 20 ˚C of F68 (grey line) and AS 100 (black line) in comparison to calculated (calc) and measured (meas) mixture curves (interrupted lines)

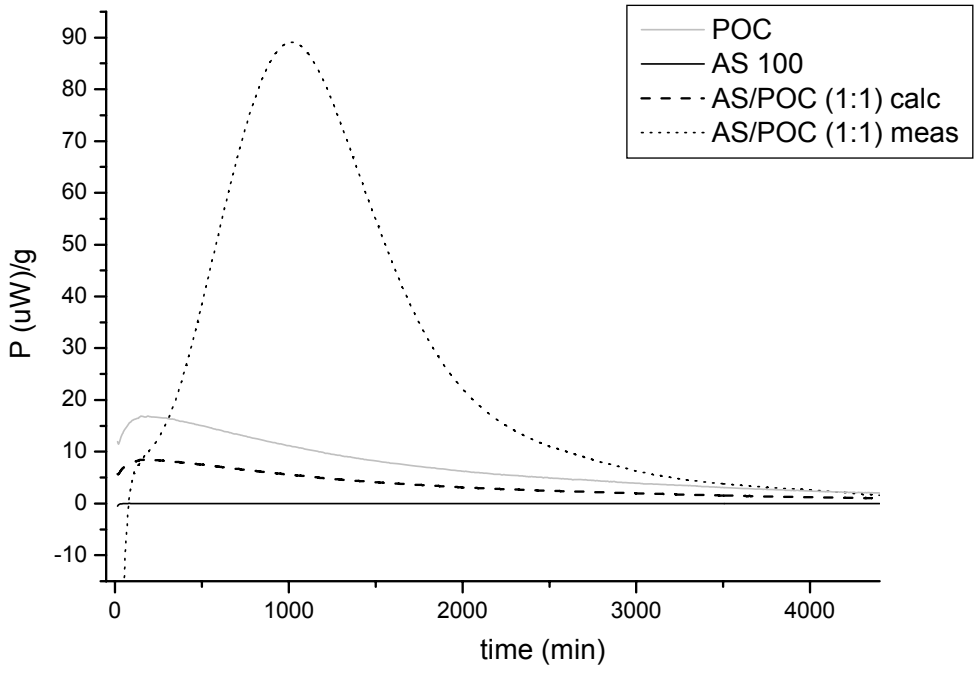

Fig. 4-10: Heat flow profiles at defined rH (100 %) at 20 ˚C of POC (grey line) and AS 100 (black line) in comparison to calculated (calc) and measured (meas) mixture curves (interrupted lines)

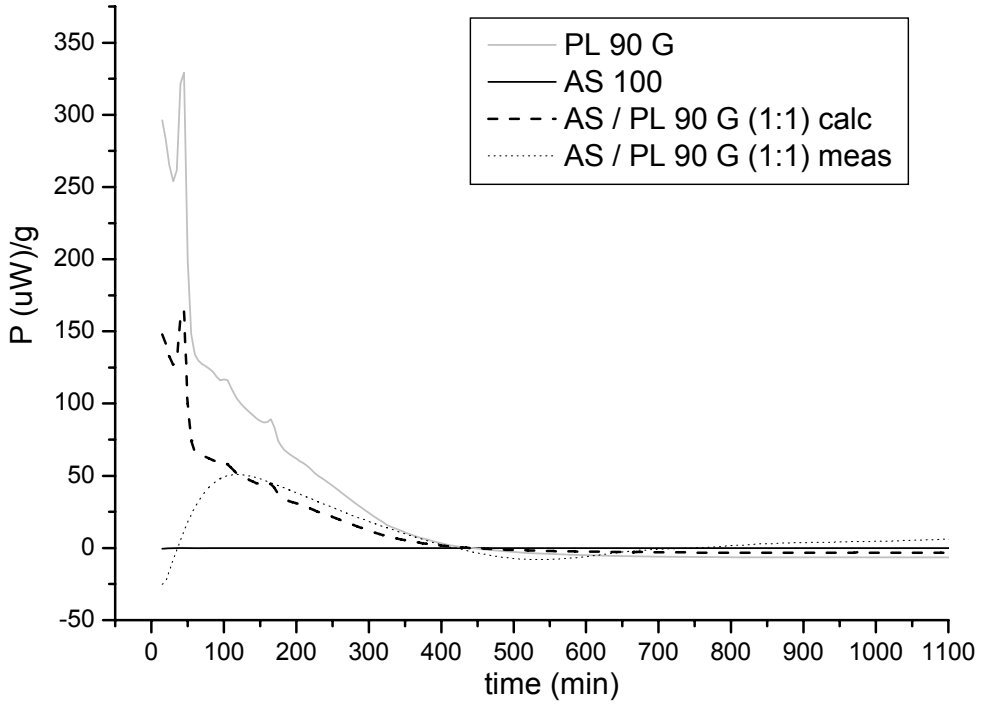

Fig. 4-11: Heat flow profiles at defined rH (100 %) at 20 ˚C of PL90G (grey line) and AS 100 (black line) in comparison to calculated (calc) and measured (meas) mixture curves (interrupted lines)

However, emulsions prepared with AS/P90G break within one week after autoclaving. Therefore AS emulsions should be prepared under aseptic conditions.

4.3.3 Dry powder emulsions

When an emulsion composed of AS 100 10.0 % (w/w) and MCT 10.0 % (w/w) (Fig. 4-12) adjusted to pH 6.5 is spray-dried (inlet 180 °C, outlet 95 °C) particle sizes get larger after spray drying and reconstituting the spray dried product (D90/ D50 1.19 µm/0.51 (before) and 3.44 µm/1.60 µm (after)).

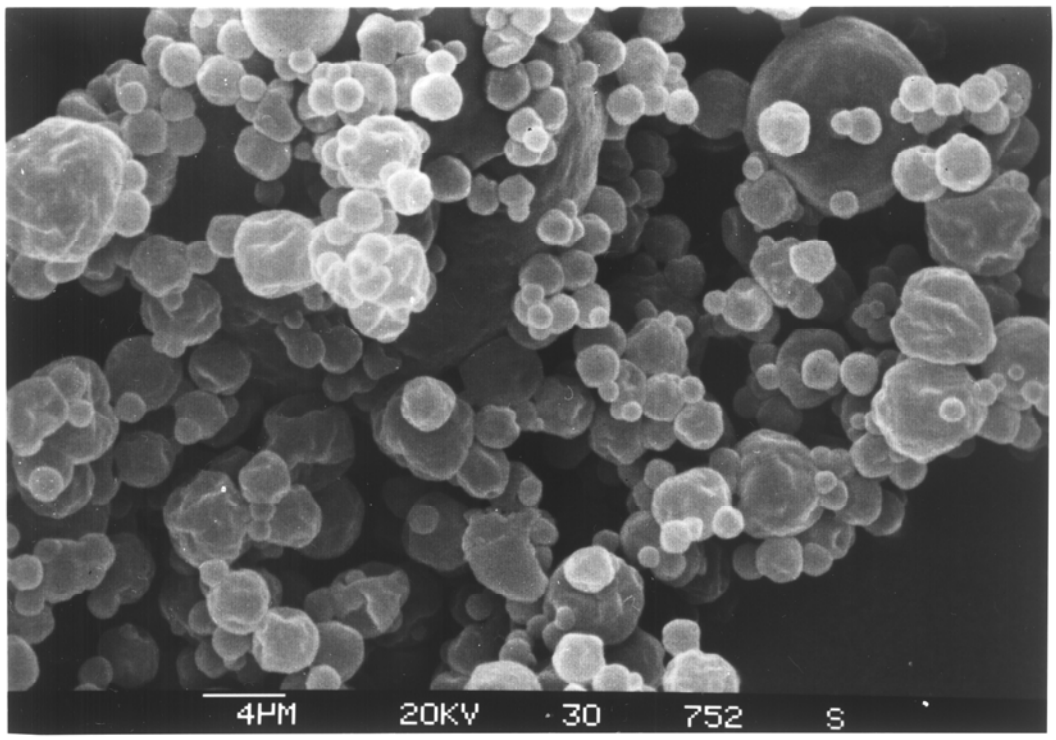

Fig. 4-12: SEM image of a spray dried AS emulsion (AS 100 10.0 % (w/w) and MCT 10.0 % (w/w)

Spray drying at the same conditions emulsions that consist of AS 25 % (w/w) and MCT 15 % (w/w), altogether a non-evaporable content of about 40 % (w/w) results in emulsions with D90/D50 about 0.82/0.45 µm (water content below 5 %) before the drying process and after reconstitution. These spray dried emulsions, reconstituted ones and dry powders stored in a tightly sealed ampoule (20 °C), are stable for at least 6 months. They can be easily redispersed prior to usage.

4.3.4 Liberation studies

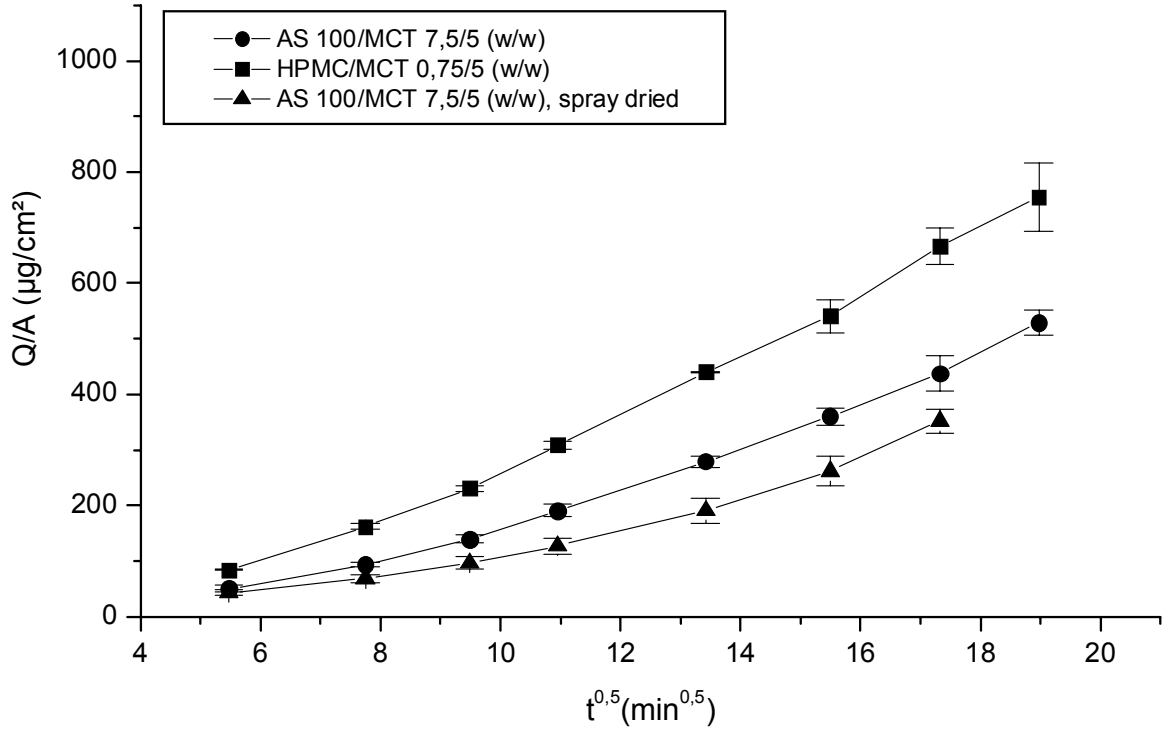

Fig. 4-13: Liberation profiles of DfNa from different emulsion systems: (circles) AS 100/MCT 7.5/5 (w/w); (squares) HPMC/ MCT 0.75/5 (w/w); (triangles) AS 100/MCT 812 7.5/5 (w/w) spray dried

AS emulsions seem to release DfNa more slowly than an HPMC emulsion. Release can be additionally decreased when the emulsion has been spray dried which may be due to a solidification of the emulsifier at the interface. This effect may benefit a sustained or controlled release of the drug at the site of application.

4.4 Conclusion

AS are polymers, which are capable of stabilizing emulsions without using classic emulsifying agents like surfactants. Since AS loses emulsifying properties at pH values above 7, emulsions should be adjusted to 6.5 to achieve an acceptable compromise between stability and ocular tolerance. However, AS formulations cannot be sterilized by

autoclaving and have to be prepared aseptically. Stability during autoclaving could not be improved by adding PL90G or co-emuslifiers, like POC or F68. Especially in the case of the latter two this was due to significant interactions, which was confirmed by IHCC measurements. Combination with DfNa revealed a low level of interaction suggesting a good compatablity.

AS emulsions can easily be spray dried and reconstituted prior to usage, whereas an emulsion with a non-evaporable content of 40 % (w/w) (AS 25 % and 15 %) proved to yield stable emulsions with comparable droplet sizes as obtained prior to spray drying. Spray dried emulsions reveal slower release profiles for DfNa as compared to non-spray dried emulsions. Therefore, considering AS, spray drying is an interesting method to formulate dry emulsions with a long storage stability and for controlled release formulations.

Thus, when administered as an emulsion, AS may be useful for both the formulation of ophthalmic and other non enteral applications.

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Part II

Cytotoxicity studies and ocular tolerance

5 The eye and ocular tolerance studies

5.1 Anatomy and physicochemistry of the eye

5.1.1 The human eye

Figure 5-1 presents a schematic view of the main anatomic elements of the human eye [Wheater et al., 1987].

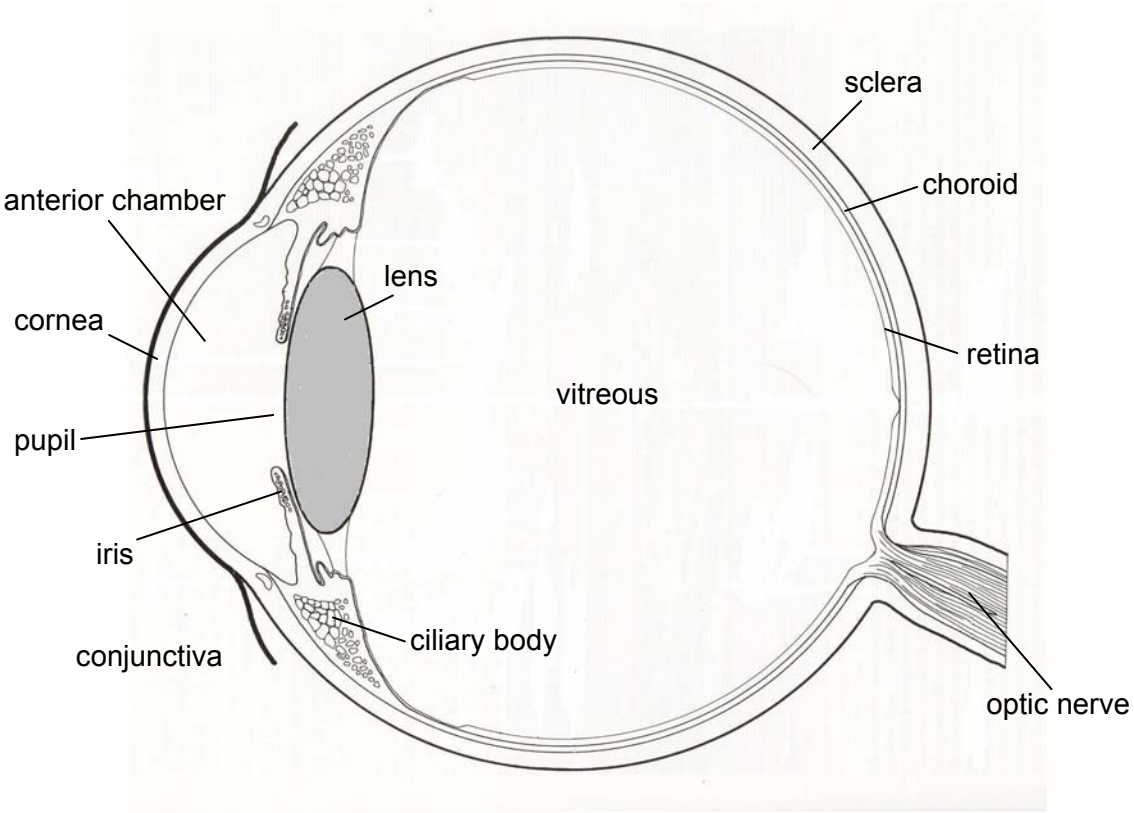

Fig. 5-1: Sagittal view of the human eye with its main anatomic elements [adapted from Wheater et al., 1987].

5.1.2 Cornea

The cornea is an important mechanical and chemical barrier, which limits the access of exogenous substances into the eye in order to protect intraocular tissues. The human cornea is a transparent, avascular tissue with a mean thickness of 500 µm in the central region becoming about 50 % thicker towards the periphery. It consists of five layers: corneal epithelium, Bowman's membrane, stroma, Descemet's membrane and endothelium (Fig. 5-2) [Hornof et al., 2004].

The corneal epithelium is composed of 5–6 layers of cells and makes up about 5 % (50-100 µm) of the total corneal thickness. It consists of a single layer of basal columnar cells, an intermediate zone (2–3 layers) of polygonal wing-shaped cells and superficial squamous cells (2–3 layers).

Tight junctions between superficial epithelial cells prevent permeation of hydrophilic drugs and water and solutes from the tear fluid into the stroma [Greaves and Wilson, 1993; Hornof et al., 2004].

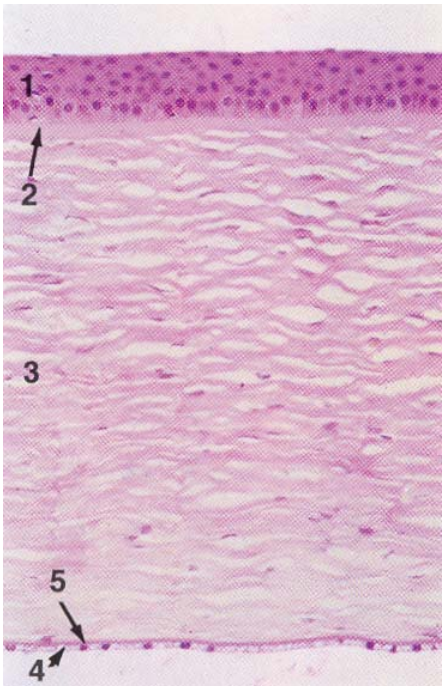

Fig. 5-2: Microphotograph of human normal cornea (haematoxylin and eosin staining, magnification x 80); (1) epithelium, (2) Bowman's membrane, (3) corneal stroma (substantia propria), (4) Descemet's membrane (5) endothelium [Wheater et al., 1987].

The corneal stroma makes up about 90 % of the corneal tissue which is bounded internally by the Descemet's membrane and the endothelium, and externally by the Bowman's membrane and the epithelium. It is composed of an extracellular matrix formed of hydrated collagens and proteoglycans. Due to its hydrophilic nature the stroma exerts a diffusional barrier only to highly lipophilic drugs [Greaves and Wilson, 1993; Hornof et al., 2004].

The corneal endothelial monolayer maintains an effective barrier between the stroma and aqueous humour. Active ion and fluid transport mechanisms in the endothelium are responsible for maintaining corneal transparency [Hornof et al., 2004].

5.1.3 Precorneal tear film

The precorneal tearfilm is a highly specialized fluid layer continuously bathing the corneal epithelium, conjunctiva and walls of the conjunctival cul-de-sac. The ocular tear fluid maintains a non-keratinized surface essential for corneal transparency and lubrication required for the movement of the lids over the globe. Abnormalities of the tear film can lead to dysfunction of the conjunctiva and eyelids as well as to loss of corneal transparency [Greaves et al., 1992].

According to the “three layers theory”, the precorneal tear film consists of a superficial lipid layer, a central aqueous layer and an inner mucus layer (Fig. 5-3).

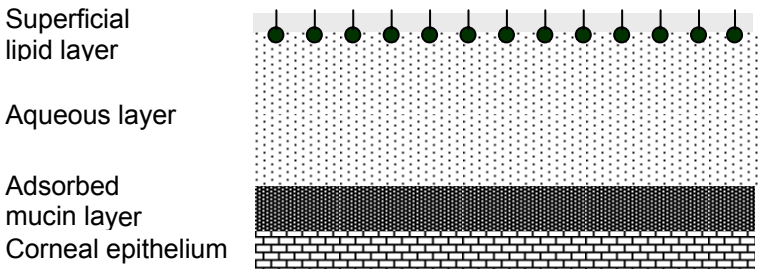

Fig. 5-3: Structure of the precorneal tear film.

The superficial lipid layer is secreted primarily by the Meibomian glands, as well as the glands of Zeiss and Moll. It consists mainly of sterol esters, triacylglycerols and phospholipids, free sterols and free fatty acids. The function of this outmost layer of the tear film is to prevent tear evaporation from the ocular surface in order to maintain physiological tear osmolality of the aqueous layer underneath.

The aqueous layer constitutes the middle layer of the tear film and is produced by the lacrimal gland and its accessory glands. The aqueous layer supplies the ocular surface with essential components including inorganic salts, glucose and urea, trace

elements, as well as vitamins (retinol, ascorbic acid), proteins and glycoproteins, including antibacterial proteins like lysozyme [Baeyens and Gurny, 1997; Qu and Lehrer, 1998]. The osmolality of the tear film is 310–334 mOsm/kg in normal eyes and is adjusted by the principal inorganic ions Na^+, K^+, Cl^-, HCO_3^-, and proteins [Dolder, 1990]. The osmotic pressure of tears is slightly higher than that of blood due to constant evaporation of water from the tear film. The pH value of tear fluid lies between 7.1 and 7.6, the surface tension between 40 and 50 mN/m and the viscosity between 1.3 and 5.9 mPa.s [Dolder, 1990].

The mucus layer, which is secreted onto the eye surface by the goblet cells, is intimately associated with the glycocalyx of the corneoconjunctival epithelial cells [Greaves and Wilson, 1993]. This layer promotes continuity of the tear film by reducing the surface tension. Mucus is the predominant component of the tear film forming the first barrier between the outside environment and the underlying corneal epithelial cells.

More recent investigations regarding the structure and composition of the tear film indicate that the “three layers theory” needs to be revised. It was found that the in vivo structure of rat tear film is composed primarily of mucus, with a lipid layer covering its surface but without a free aqueous layer [Chen et al., 1997]. Estimates of the tear film thickness, found in the literature, varies from approximately 4 µm to 40 µm and the real value remains controversial [Wang et al., 2003].

5.2 Ocular tolerance studies

5.2.1 Ocular toxicity

The eye possesses effective protective mechanisms like lacrimation, blinking, tear drainage and tear turnover, that prevent chemicals and other irritative objects/ environmental pollutants from remaining too long on the eye surface and maintain an adequate tear film. The cornea is densely innervated, which contributes to the immediate blinking reflex and retraction of the head upon irritation.

Nevertheless, highly potential drugs or toxic drugs/excipients that are applied on the eye may cause different toxic effects depending on the application site and whether the specific substance is absorbed or not. Local toxicity may result from a direct contact of a chemical with the eye and may be limited to superficial tissues like the cornea and conjunctiva (superficial local toxicity) or affect deeper tissues like the iris, crystalline lens or even the retina (deep local toxicity) [Furrer, 1999].

Beside local toxicity also systemic toxic effects may occur if the applied substance is absorbed by the conjunctival vessels or by the alimentary tract after passing through the nasolacrimal system. On the other hand the eye may also be a target for toxicity if a drug or its metabolites reach the eye through the systemic circulation [Furrer, 1999].

Ocular tolerance is the ability of ocular tissues to bear a given dose of a chemical without showing evidence of intoxication [Etter and Mayer, 1985]. While *eye corrosion* is an irreversible ocular tissue damage at the site of contact resulting in lesions of deep ocular tissues, *eye irritation* can be defined as a reversible morphological or physiological damage to the eye and its surrounding mucus membranes after direct exposure to a material on the surface of the anterior eye segment. The latter damage consists generally of non-immunological inflammatory reactions [Furrer, 1999].

Ocular safety tests implicate pathological modifications affecting cornea, conjunctiva and iris. Any lesion causing a disruption of anatomical features (neovascularization, pigmentation, scarring) or a swelling of the cornea will alter corneal transparency resulting in visual impairment [Meek et al., 2003]. Corneal injuries comprise many various forms such as dryness (keratoconjunctivitis sicca), neovascularization and opacity [Kast 1991; Van Winkle, 1991]. The severity ranges from abrasion (local loss of epithelial cells), erosion (loss of several epithelial layers) and ulceration (loss of epithelial and stromal layers) to perforation (damage of the endothelium) [Furrer, 1999]. Ocular irritation concerning the conjunctiva appears as an inflammatory reaction called conjunctivitis. The iris, being a vascular muscular structure, forms the pupil and regulates the amount of light that reaches the retina. Iridial irritation may be

associated with inflammation signs, like aqueous flare and aqueous cellular reactions, and lack of reaction to light [Furrer, 1999].

5.2.2 Methods to investigate ocular tolerance

5.2.2.1 The Draize test for ocular irritation

The evaluation of ocular toxicity is not only important to assess side effects of drugs and excipients in pharmaceuticals, but also crucial to assure a safe use of cosmetics, consumer products and industrial products.

Since the 1940's eye irritation traditionally has been evaluated using the Draize in vivo rabbit eye-irritation test [Draize et al., 1944]. This method is based on the macroscopic observation of the treated eyes of albino rabbits, after 0.1-ml (or weight equivalent) sample of test material is placed into their lower conjunctival cul-de-sac. The untreated eye is used as a control. Responses (e.g. injuries, redness, opacity, swelling, discharge) of the cornea, conjunctiva and iris, are graded at standard times, generally from 1 to 35 days after dosing. The tissue grades are combined into a weighted score following a scoring system that converts the qualitative observations of pathological evidence in quantitative data [Furrer 1999, Kulkarni et al., 2001].

The in vivo rabbit eye-irritation test is very simple, easy to conduct and requires no special instruments. Albino rabbits are easy to handle, available, have a relatively large ocular surface and the lack of pigmentation enables a good observation, especially of the iris. But despite of its widespread use it has frequently been criticized not only on behalf of animal welfare. Regarding the methodology the difficult interpretation of the results and the subjective scoring may lead to intra-/interlaboratory variability which in turn leads to a limited reproducibility. A third important point of major criticism is the applicability to the human eye. A summary of the differences in ocular physiology between man and rabbit, mainly influencing preocular loss and absorption/permeation, is given in table 5-1. Selecting the rabbit as an animal model is only appropriate if the results obtained are predictive of the behaviour in humans [Reddy and Ganesan, 1996].

Tab. 5-1: Comparison of selected anatomical and physiological differences between the rabbit and human eye [adapted from Greaves et al., 1992; Reddy and Ganesan, 1996]

Parameter	Rabbit	Human
Tear volume	5–10 µl	5–10 µl
Tear flow	0.5–0.7 µl/min	0.5–2.2 µl/min
Tear turnover rate	8 %/min	16 %/min
Tear film pH	8.2	7.4
Drainage of instilled fluid	Slow	Fast
Blink rate	4–5/h	15–20/min
Corneal thickness	0.4 mm	0.5 mm
Corneal diameter	15 mm	11 mm
Corneal permeability	high	low
Bowman's membrane	Absent	Present
Nictitating membrane	Present	Absent

For the reasons mentioned considerable work has been done to develop alternative methods to study ocular safety.

5.2.2.2 Alternative in vitro tests

In vitro methods allow a large number of measurements to be performed, easily controllable and reproducible, require less time, are painless and relatively inexpensive. The following paragraphs shortly outline some commonly used in vitro methods to evaluate the toxicity potential of chemicals.

5.2.2.2.1 Isolated ocular tissues

Corneal injury represents only one endpoint of in vivo eye irritancy, but it makes by far the greatest contribution to the maximum total Draize score. Therefore, isolated corneas of either bovine or porcine origin have been suggested as suitable models in

risk assessment. Irritation endpoints are opacification, coloration to fluorescein or enhanced permeability to a fluorescent dye [Herzinger et al., 1995] epithelial detachment or loss of corneal integrity [Ellingson et al., 1992; Baydoun et al., 2004]. Besides problems of availability and viability of the isolated organs, responses to toxic insults achieved with this method are difficult to quantify.

5.2.2.2.2 Fertilized hen's egg

The chorioallantoic membrane (CAM), an extraembryonal circulatory system of the chicken embryo, is a highly vascularized, stratified tissue, which originally has been suggested to provide a model for the conjunctival tissue of the eye. The HET (Hen's Egg Test or Hühner-Embryonen-Test)-CAM test is a rapid, sensitive and inexpensive toxicity test and can give information on embryotoxicity, teratogenicity, systemic and immunopathological effects and on mucous-membrane irritation potencies of chemical substances [Lüpke, 1985; Märtins et al., 1992]. Irritation endpoints are lesions, coagulation and hemorrhage. Toxic potential can be expressed in terms of the concentration of test material that elicits a positive response in 50 % of the eggs. A positive response is considered to be a membrane lesion with a maximal diameter of more than 2 mm [Herzinger et al., 1995].

Limitations of this assay are the bad predictability of potential in vivo irritation and that it does not permit the evaluation of sensory irritation or discomfort since the model used is no innervated tissue [Märtins et al., 1992].

5.2.2.2.3 Cultured cells

One major advantage of cell culture systems lies in the possibility that cells can be obtained from the particular target organ, like the cornea, and from human origin [Reichl et al., 2004]. The number of surviving cells, counted e.g. in a hemocytometer or in an electronic cell counter, after exposure to the test material is probably the most simple endpoint. Alternatively the uptake and accumulation or enzymatic reduction of a dye, like neutral red or MTT, respectively, by viable cells can be

determined [Sina et al., 1995; Imbert and Cullander, 1997]. Like tests on isolated tissues assays with cultured cells are still not fully comparable to the in vivo situation. Tearing and blinking, for instance, limit the contact time of the test material and the cells, which may account for false positives achieved in vitro [Sina et al., 1995]

5.2.2.2.4 Red blood cell haemolysis

The haemolytic potential of surfactants was suggested to be indicative of their in vivo ocular irritancy [Pape et al., 1987; Pape and Hoppe 1990]. Red blood cells are readily available, either from animal or human origin, and easy to handle. Tensides possess a high affinity to react with cell membranes, affect functional and structural units of proteins and change membrane permeability. Using red blood cells the amount of released haemoglobin, due to membrane damage, can easily be detected and quantified spectrophotometrically [Bock and Müller, 1994; Jumaa et al., 2002]. The assay is rapid, inexpensive and yields reproducible results. The haemolysis assay is not proposed as an extensive alternative to the Draize test, but may provide useful information on tissue compatibility of newly developed surfactants or tenside formulations. This may help reduce the number of animals needed to perform a Draize test.

5.2.2.3 Alternative in vivo methods

Despite the advantages in vitro methods offer they are unable to reproduce the complete irritation response observed in the vivo situation. Since the eye is a very complex and sensitive organ one in vitro method cannot possibly assess all effects of test materials and the reaction of the living eye when exposed to them [Furrer, 1999]. None of the currently available in vitro methods has proven to be a valid substitute for in vivo ocular safety tests and at the most help to pre-screen new drugs/excipients. Only in vivo testing provides the full spectrum of potential reactions and enables a more reliable prediction of human eye irritancy.

There is a number of in vivo methods like (a) pachymetry (measurement of corneal thickness due to edema or ulceration, (b) tonometry (measurement of intraocular pressure after exposure to an irritant, (c) wound healing studies (objective observation of the delayed recovery time after contact with an irritant) or (d) the increase of corneal permeability to a marker dye as a consequence of tissue lesions [Furrer, 1999].

Conventional light microscopy cannot be used for the in vivo examination of the eye. Especially thick specimens with overlapping structures produce an out-of-focus blur which reduces the contrast and resolution of the image. By contrast, a confocal microscope is a type of microscope in which a thick object like the cornea is illuminated with a focused spot of light. It permits the investigation of optical sections of relatively thick specimens (> 10 µm). Since confocal microscopy suppresses the out-of-focus blur, sharp three-dimensional images can be obtained. As described already elsewhere [Furrer et al., 1997] this is accomplished by two features as follows:

(a) The arrangement of the condenser (part of the illumination system) and the objective lens (part of the detection system): both have the same focal distance and are disposed symmetrically with respect to the focal plane (confocal arrangement).

(b) There is a spatial filter (in the form of a pinhole, diaphragm or slit) placed in front of the detector to ensure that only light from coming from the focal plane is collected.

Either a white light source with a second spatial filter placed in front of it or a laser beam, which is already tightly bundled, is used to restrict the illumination to a small spot. This arrangement improves resolution by about 40 % but since the field of view is restricted to a single point the full image needs to be generated by scanning the specimen. This is carried out by moving the focus, both vertically and horizontally in a raster pattern along the optical axis. The two-dimensional images of a series of focal planes are subsequently transferred into a stack; this addition provides a three-dimensional image [Furrer et al. 1997].

The confocal laser scanning microscope (CLSM) uses a laser beam instead of white light (Fig. 5-4). With the CLSM, only a single spot of laser light scans periodically the stationary object field. The light reflected, transmitted or emitted by the specimen, is then oriented to the detector by a dichroic mirror. The detector, generally a photomultiplier, converts the light to an amplified signal which can be processed further. This technique allows the rapid reconstruction of clear three-dimensional images but no visualization in true colours [Furrer et al., 1997].

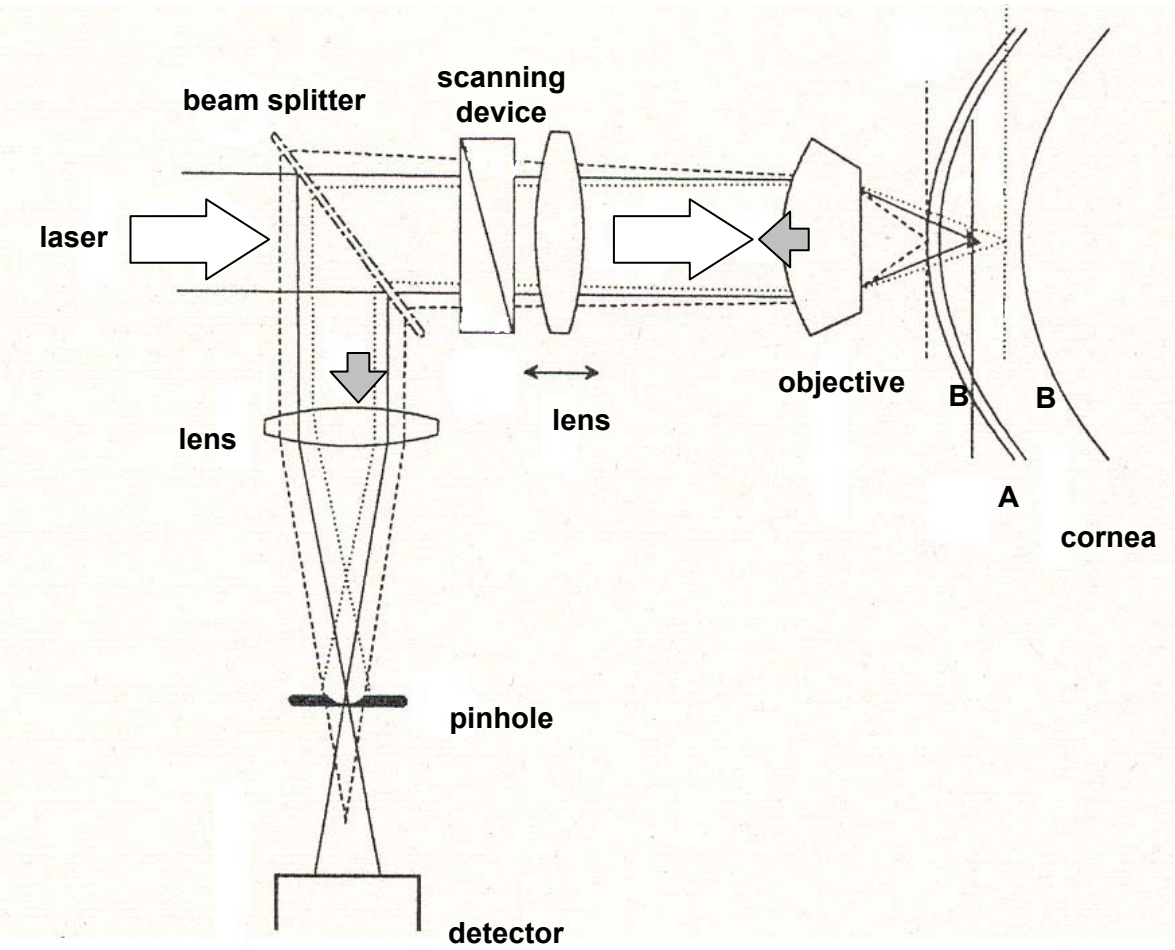

Fig. 5-4: Schematic representation of a confocal laser scanning microscope [adapted from Furrer et al., 1997]: A laser beam is moved by a scanning device and focused on the cornea by an adjustable tube lens. The reflected or induced light is directed into a detector by a beam splitter (dichroic mirror). In-focus light (A) from the stromal tissue passes through the pinhole whereas out-of-focus light (B) remains undetected.

The combination of the ophthalmoscope, which has originally been developed for the examination of the retina, with confocal and laser technologies provides a non-invasive tool with a high resolution as achieved with electron microscopy to

investigate the anterior part of the eye [Furrer, 1999]. The confocal laser scanning ophthalmoscope (CLSO) coupled with a labelling technique of corneal lesions using fluorescein, enables an objective, reproducible, sensitive and simple evaluation of in vivo ocular toxicity [Maurer et al., 1999; Furrer et al., 2000]. CLSO is applicable to animals and directly to humans [Furrer, 1999].

6 *n*-octenylsuccinate starch: Evaluation of ocular tolerance

Parts published in:

Baydoun L, Furrer P, Gurny R, Müller-Goymann CC. **2004**. *New surface-active polymers for ophthalmic formulations*: *evaluation of ocular tolerance.* Eur J Pharm Biopharm 58: 169–75

6.1 Introduction

Polymeric hydrogels have been widely used to increase viscosity and consequently retention time of eye drops on the ocular surface in order to increase intraocular drug levels. The most widely used polymers include cellulosic derivatives, poly(vinyl alcohol), sodium hyaluronate, and carbomer [Felt et al., 2002]. The use of *n*-octenylsuccinate starch has been described for the formulation of anti-inflammatory eye drops [Baydoun and Müller-Goymann, 2003].

Amphiphilic starch of the *n*-octenylsuccinate starch type (AS) is a chemically modified waxy maize starch gained by substituting hydrophilic starch moieties by lipophilic *n*-octenylsuccinic acid groups [Gers-Barlag and Müller, 2002; Järnström et al, 1995]. Consequently, the starch acquires emulsifying properties due to feasible formation of hydrophobic-hydrophobic interactions. AS is used in the food and cosmetic industry [Viswanathan, 1999; Wesslén and Wesslén, 2002] as an alternative to traditional stabilizers such as spray dried gum acacia, vegetable proteins, lecithin, or gelatin [Järnström et al, 1995; McGlinchey, 1997].

Sodium diclofenac, solubilized in an AS dispersion, has shown improved corneal permeation behaviour as compared to tested marketed eye drop preparations, where the drug is solubilized by means of polyoxyethylene castor oil (POC) [Baydoun and Müller-Goymann, 2003]. Due to AS's emulsifying properties, it is mandatory, when its use is intended for the ocular route, to assess in vivo its ocular tolerance. Indeed, surfactants or preservatives with surfactant properties used in eye drops are known to cause varying ocular irritations by mechanisms such as association with biological membranes resulting in changes to surface charge, alteration of barrier functions, or denaturation and cell death [Furrer et al., 2000; Patlewicz et al., 2000].

Currently, the Draize rabbit eye test is used to assess ocular irritancy. However this methodology has been criticized for its lack of objectivity and sensitivity. More recently, alternatives have been proposed to replace the Draize test including: light microscopy and confocal microscopy. These approaches have been shown to objectively quantify surfactant-induced eye irritation in the rabbit [Jester et al., 2001].

The aim of the present study was to compare, with respect to their ocular tolerance, different AS types of unknown molecular weights and diverse emulsifying properties.
Both types, AS 100 and 300, provided by different manufacturers, are identical in chemical structure but may differ in degree of substitution (ds), which does not exceed 3 %. They are won from waxy maize starch, which is chemically modified and hydrolysed, by controlling the viscosity, to reduce the molecular weight of the starch molecule. The investigated AS types mainly differ in molecular weight and molecular weight distribution. Since, it was found that increasing the ds does not necessarily improve the emulsifying properties of AS [Viswanathan, 1999] both AS types were characterized by performing viscosity, vapour pressure and surface tension measurements in order to gain information on molecular weight and molecular weight distribution, which greatly influence emulsion formation and stability [Baydoun and Müller-Goymann, 2001].

To evaluate the preparations' irritation potential, in vitro tissue acceptability studies on human erythrocytes and excised porcine cornea and in vivo ocular tolerance tests were carried out in rabbit eyes. After applying AS dispersions and emulsions at different concentrations in vivo to the rabbits' corneas, tissue damage was assessed using a confocal laser scanning microscope following fluorescein labelling. Additionally, the tested formulations were incubated with human erythrocytes and excised porcine corneas. The amount of released haemoglobin due to membrane damage was quantified by UV detection and histological cross sections of treated pig eye material were evaluated by light microscopy for pathological modifications caused by an irritant substance.

6.2 Materials and methods

6.2.1 Materials

Medium chain triglycerides (MCT 812) and thimerosal were purchased from Synopharm (Barsbüttel, Germany), sorbitol from Hänseler AG (Herisau, Switzerland) and sodium hydroxide from Amtech-Chimie SA (Lausanne, Switzerland). Sodium fluorescein was obtained from Reactolab (Servion, Switzerland). Sodium chloride, potassium dihydrogen phosphate and disodium hydrogen phosphate (all pro analysi), purchased from Merck (Darmstadt, Germany), were used to prepare isotonic phosphate buffer pH 7.4 (PBS) according to the German Pharmacopoeia (DAB 2001); all substances used were of analytical or pharmacopoeia grade.

AS type 100 and type 300, both emulsifying starches, were supplied by National Starch & Chemical (Manchester, United Kingdom) and Roquette frères (Lestrem, France), respectively. Double-distilled water was used for all preparations.

6.2.2 Experimental methods

6.2.2.1 Flow measurements

Flow measurements were performed to asses the systems' viscosities. The method applied is described in paragraph 2.2.2.1.

6.2.2.2 Osmolality

Osmotic activities of investigated preparations were analysed by vapour pressure measurements at 37 °C according to chapter 2, paragraph 2.2.2.2.

6.2.2.3 Surface tension

Surface tension measurements were carried out as described in paragraph 2.2.2.3.

6.2.2.4 Preparation of AS formulations for in vitro and in vivo tolerance tests

The AS dispersions (AS D) investigated contained 2 % and 15 % (w/w) AS of both types. AS moisture contents were previously determined by thermogravimetry (Thermal Analysis System SSC 5200, Software: MAS 5700 MA-Station Version 3.2, SSC 5200H Disk-Station Version 3.2, Version/Typ: DSC 220C, Seiko Instruments, Tokyo, Japan). AS emulsions (AS E) were stabilized with 15 % (w/w) AS 100/300. The lipophilic phase consisted of MCT 812. Oil-in-water (o/w) emulsions (10 % (w/w) oil phase) were prepared by dissolving AS in cold water and adding it to the oil phase using an Ultra-Turrax (Janke & Kunkel, Staufen, Germany). The pre-emulsions were passed through a high pressure homogenizer (Niro Soavi, type: Panda, Parma, Italy) six times at room temperature applying a pressure of 400 bar (AS 100) and 300 bar (AS 300) to achieve submicron emulsions (D90 around 1 µm). Particle size distribution was analysed by laser diffraction (Mastersizer MS 20, Malvern, Worcs, United Kingdom) and calculated by Malvern SB 09 software using the Mie approximation.

pH values of all formulations tested were adjusted to 6.5 using a 0.1 N sodium hydroxide solution as AS loses its emulsifying properties at pH values higher than seven, where emulsions tend to break quickly [Baydoun and Müller-Goymann, 2001]. Osmolality was adjusted with sorbitol using a calibrated vapour pressure osmometer (Wescor 5500, Baumann-Medical, Wetzikon, Switzerland) and ranged between 280 and 330 mOsm/kg.

6.2.2.5 In vitro investigation: Red blood cell haemolysis and influence on porcine cornea integrity

Red blood cells were obtained from human blood (27-year-old female with common blood chemistry) by centrifugation (5 min, 1000 × g). After the supernatant plasma was removed the erythrocytes were washed three times with PBS 7.4 in order to remove serum proteins. Using PBS 7.4 an erythrocyte stock dispersion with a fixed haemoglobin concentration was prepared. The stock dispersion was refrigerated and kept no longer than 24 hours.

One hundred microlitres of the stock dispersion were added to 1000 µl sample, well shaken and incubated at 37 °C for different time periods (15 min, 30 min, 1 h). The samples were shaken every 5 min. After centrifugation (3 min, 750 × g), to remove intact erythrocytes and debris, 100 µl of the supernatant were added to 2000 µl of an ethanol/HCl mixture (40 parts of ethanol 99 % (v/v) and 1 part of hydrochloric acid 37 % (w/v)) and centrifuged again (3 min, 1000 × g). The ethanol/HCl mixture avoids haemoglobin precipitation [Jumaa and Müller, 2000].

The absorption of the subsequently achieved supernatant was measured by spectrophotometry at 398 nm against blank samples (ethanol/HCl mixtures containing the same amounts of AS and drug as the samples).

Results were set in relation to control samples of 0 % lysis in PBS 7.4 and 100 % lysis in double-distilled water. Total haemolysis must show an absorption of about 2.0 ± 0.2 in order to obtain linearity in absorption concentration dependence.

To examine the influence on corneal structure and integrity, cornea was removed from fresh pig eyes [Baydoun and Müller-Goymann, 2003] and incubated at 37 °C for 2 h in the AS formulations. PBS and a sodium dodecylsulfate (SDS) solution in PBS 0.1 % (w/w) were taken as references. Corneas were incubated for 0.5 h in SDS 0.1 % (w/w).

After incubation, the corneas were washed with PBS, and immediately fixed with a formalin solution 8 % (w/w). The material was dehydrated with an alcohol gradient, put in melted paraffin and solidified in block form. Cross sections (< 1 µm) were cut, stained with haematoxyline and eosine (H & E), blinded and microscopically observed for pathological modifications (n = 3) in co-operation with a pathologist.

6.2.2.6 In vivo evaluation using confocal laser scanning ophthalmoscopy

New Zealand albino rabbits of either sex, weighing between 4 and 5 kg were separately kept in an air-conditioned, illumination-controlled room at 19 ºC ± 1 ºC and

a relative humidity of 50 ± 5 %. They were fed a standard pellet diet and water ad libitum. All animals were healthy and free of clinically observable abnormalities.

The experiments of the present investigation were run in accordance with the Association for Research in Vision and Ophthalmology (ARVO) resolution on the humane handling of animals in ophthalmic and vision research and were approved by the local ethics committees for animal experimentation.

25 µl of each tested formulation were repeatedly applied onto the rabbit's right cornea throughout three days, every 2.5 h four times per day and once on the fourth day right before the microscopy experiment.

The fluorescent images of the treated rabbit corneas were visualized by CLSO using a previously described optical device [Furrer et al. 2000]. The total areas of corneal lesions were quantified by an image processing system [Furrer et al. 2000] and expressed in percent of the total corneal surface. Data were statistically compared applying the Student's t-test (unpaired samples). A probability level of 0.05 was chosen for all comparisons. Calculations were made with a Microsoft Excel 7.0 program.

6.3 Results and discussion

6.3.1 Characterization of AS types

Investigations were performed with two AS types, AS 100 and AS 300, which are characterised in chapter 2.

The specific characteristics of the different AS formulations evaluated in vivo and in vitro for ocular tolerance are summarized in table 6-1.

Tab. 6-1: Characteristics of different AS formulations (D = dispersion, E = emulsion) assessed for ocular tolerance, [a] osmolality adjusted with sorbitol, [b] not measured, mean value (n = 3).

		Osmolality (mOsm/kg)	Viscosity (mPa s)
AS 100	D 2 %	299[a]	n.m.[b]
	D 15 %	326	15.9
	E 15 %	320	40.0
AS 300	D 2 %	282[a]	n.m.[b]
	D 15 %	299[a]	7.4
	E 15 %	308[a]	21.2

6.3.2 In vitro evaluation of tissue acceptability

Haemolysis of the AS 100 and AS 300 systems tested do not exceed 1 % even after one hour of incubation. The results achieved with different AS types (Fig. 6-1) do not differ significantly.

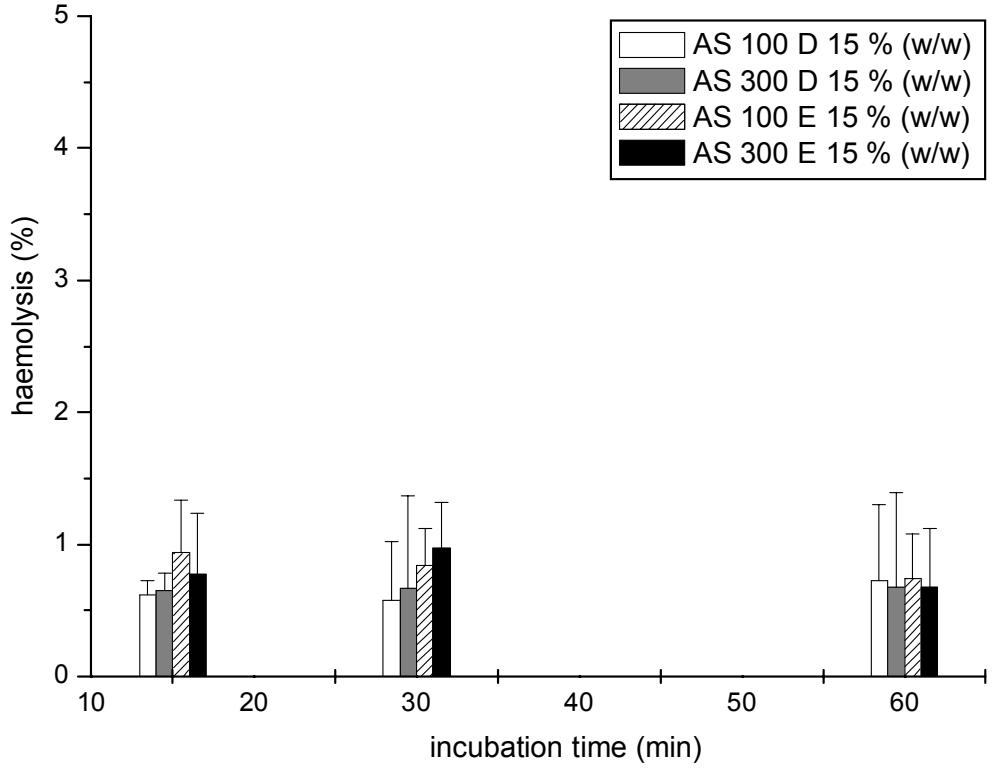

Fig. 6-1: Haemolytic activities of different AS formulations (D = dispersion, E = emulsion) depending on incubation time (37 °C). Mean ± SD (n = 3).

Figs. 6-2 a – c present corneal cross sections after incubation of freshly excised pig corneas with various preparations to investigate their influence on corneal cell structure and tissue integrity. After incubation in a physiological phosphate buffer solution pH 7.4 (Fig. 6-2 a) epithelium (EP) and stroma (ST) structure is maintained. Typical stratified epithelial layer can be recognised by the basal columnar cells and the squamous surface cells appearing with a bulge at the nuclei (NU). When the corneal epithelium is exposed to an irritant, like SDS (Fig. 6-2 b), previously narrow intercellular spaces are clearly widened, cells and nuclei are deformed and superficial epithelial cells are detached from tissue assembly.

Treating corneas with the tested AS formulations as exemplified in Fig 6-2 c, showing a cornea cross section after incubation in AS 100 D 15 %, leaves corneal structure and integrity visibly unaffected. Both haemolysis and histologic experiments reveal a good AS tissue acceptability.

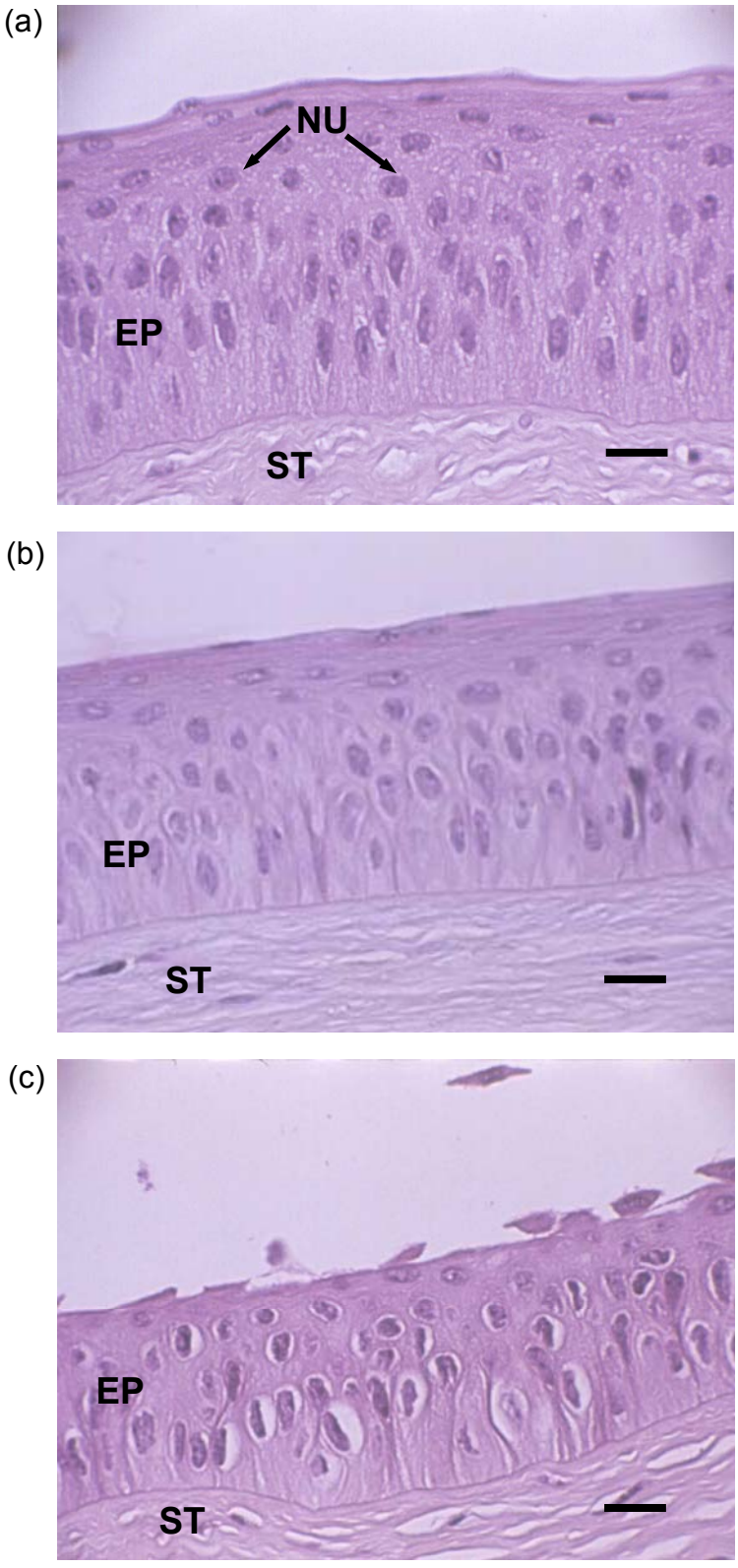

Fig. 6-2: Histologic cross sections of excised porcine cornea showing epithelium (EP) and stroma (ST), stained with hematoxylin-eosin (scale bar 20 µm) after incubation at 37 °C with: (a) Isotonic phosphate buffer (PBS) pH 7.4 (2 h) NU = nuclei; (b) Sodium dodecylsulfate (SDS) solution 0.1% (0.5 h) (c) AS 100 dispersion 15% (w/w), pH 6.5 (2

6.3.3 In vivo evaluation of ocular tolerance

The extent of corneal surface damage caused by repeated instillation of AS preparations is shown in Fig. 6-4. Two different AS types with characteristics mentioned above were tested in comparison to data achieved with a physiological sodium chloride solution [Furrer et al., 2002]. Dispersions (D) containing 2 and 15 % (w/w) AS and emulsions (E) stabilized with 15 % (w/w) AS were evaluated for ocular tolerance (Tab. 6-1).

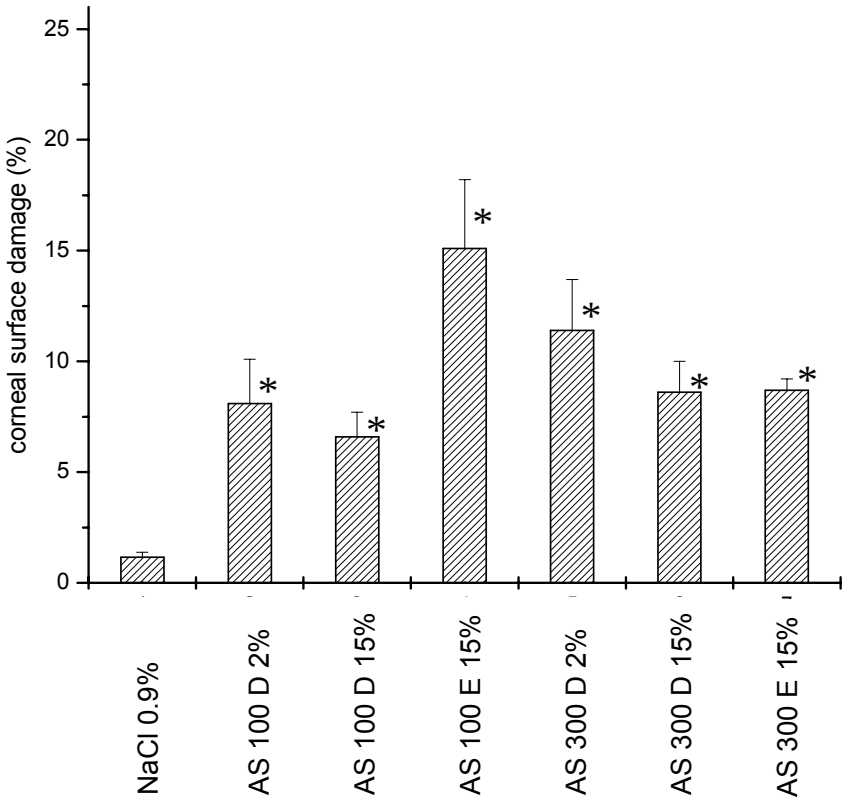

Fig. 6-3: Extent of corneal surface damage (%) in rabbits caused after instillation of various AS formulations (D, dispersion; E, emulsion) compared to a sodium chloride solution (0.9%). Mean value ± SD (n = 3 – 4), Student's t-test (unpaired samples), *P < 0.05 (significant difference)

Due to a detectable surface activity, all tested AS systems showed significantly higher corneal surface damage, ranging from 6.6 to 15.1 %, as compared to a physiological saline solution (Fig. 6-3), which causes about 2 % of damaged corneal surface. These damaged areas in the case of a saline solution are a result of physiological desquamation observed even in the healthy eye [Furrer et al., 1999]. Nonetheless, the total of fluorescent areas with AS treatment, representing harmed corneal tissue, never exceeded 19 % (Fig. 6-3). This indicates, according to a previously established scale [Kälin, 1994], a good tolerance for all AS preparations respecting the tested concentrations.

With regard to dispersions, Fig. 6-3 reveals that raising the AS concentrations from 2 to 15 % does not necessarily lead to a greater damage of corneal area. Both polymer types, AS 100 and 300, showed no significant differences. When comparing D 2 % with D 15 %, no significant increase of irritation is observed. In contrast, results obtained with preservatives and absorption enhancers have demonstrated the influence of the agents' quantity on corneal injury [Furrer et al., 1999; Furrer et al., 2002].

Although AS 100 has more pronounced surface properties than AS 300, drawn from data of surface tension measurements and emulsion formation and stability [Baydoun and Müller-Goymann, 2001], both types reveal no notable differences (Tab. 6-2) when instilled in equal concentrations independent of the sort of formulation. The degree of polymerization, or in terms of starch derivatives the dextrose equivalent (DE) value, seems to have an effect on emulsifying properties but not on the extent of ocular irritation.

Emulsions as carrier systems for ocular administration offer the possibilities of incorporating lipophilic or difficult-to-solubilize and -stabilize drugs, such as sodium diclofenac [Bock et al., 1994]. Ophthalmic emulsions with higher viscosities may extend pre-ocular retention times while, on the contrary, often a temporary blurred vision appears. Reports on the haemolytic behaviour of surface active agents have shown that erythrocyte lysis could be significantly decreased when the lytic agent was incorporated into an emulsion system [Jumaa and Müller, 2000]. This membrane protective effect described [Jumaa and Müller, 2000] could not be confirmed in eye

tolerance evaluation by confocal laser scanning microscopy. While the effects caused by an AS type 300 dispersion and emulsion (D/E 15 %) are comparable, AS 100 imparts visible differences between D and E 15 % (Fig. 6-3, Tab. 6-2). Figs. 6-4 a–c show fluorescent images of three different AS preparations, D 2 and 15 % and E 15 %. Damaged cornea tissue occurs as bright regions resulting in a total of 7.45 % (Fig. 6-4 a), 8.5 % (Fig. 6-4 b) and 15.1 % (Fig. 6-4 c).

Tab. 6-2: Statistical comparison of ocular irritation caused by AS formulations using the Student's t-test (unpaired samples, $P < 0.05$). $P < 0.05$ = significant difference, n.s. = no significant difference, ▨ = not compared.

Formulation/ concentration		AS 100			AS 300		
		D 2 %	D 15 %	E 15 %	D 2 %	D 15 %	E 15 %
AS 100	D 2 %		n.s.	▨	n.s.	▨	▨
	D 15 %	n.s.		P < 0.05	▨	n.s.	▨
	E 15 %	▨	P < 0.05		▨	▨	n.s.
AS 300	D 2 %	n.s.	▨	▨		n.s.	
	D 15 %	▨	n.s.	▨	n.s.		n.s.
	E 15 %	▨	▨	n.s.	▨	n.s.	

AS emulsions show, like AS dispersions, Newtonian flow behaviour (see results 2.3.1), while AS 100 E 15 % produces a comparably high viscosity of 40.0 mPa s (Tab. 6-1). On the other hand, emulsions may break on contact with the cornea and lacrimal fluid which leads to higher local emulsifier concentrations. Both effects may explain the increased but still acceptable irritancy.

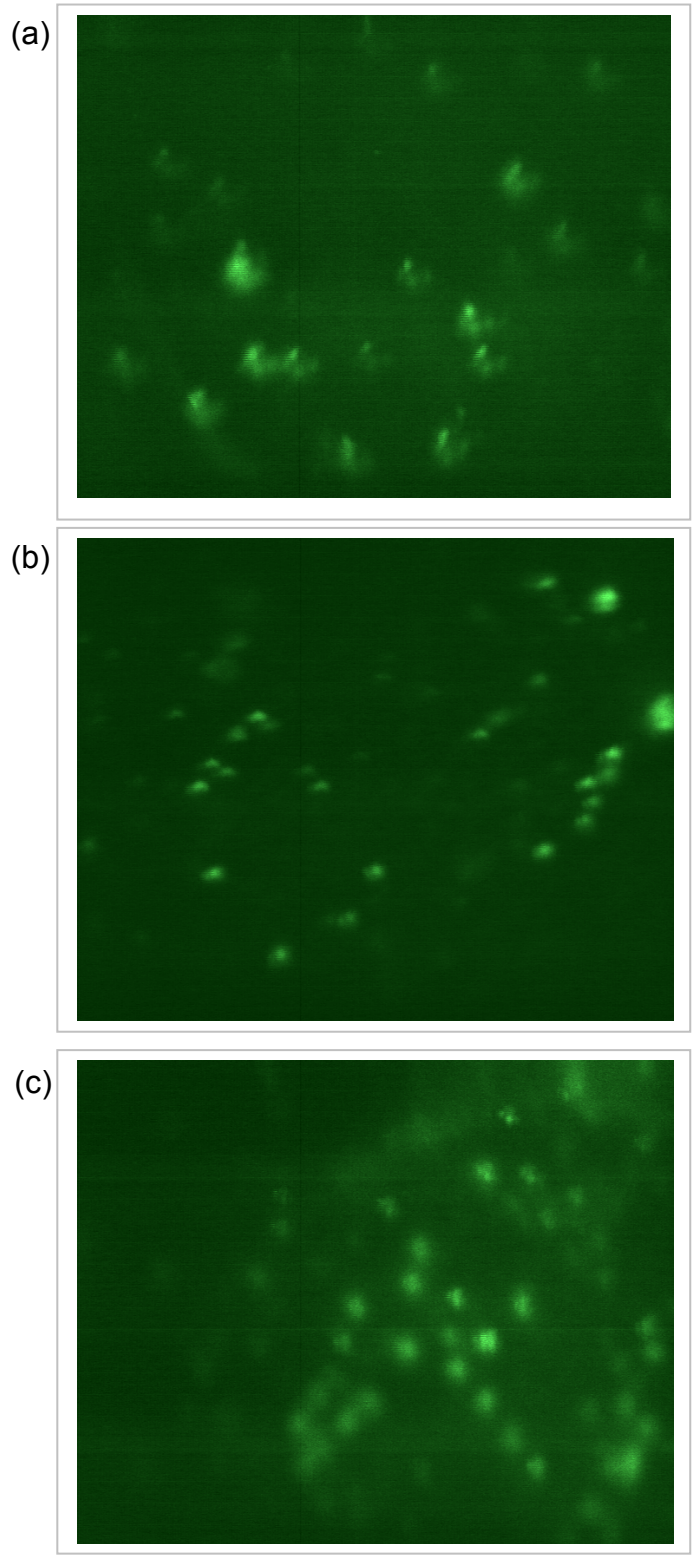

Fig. 6-5: Fluorescent images of rabbit corneas illustrating corneal lesions (bright spots) after repetitive instillation of 3 different AS 100 preparations (S, solution; E, emulsion).
(a) AS 100 S 2 % (w/w) represents 7.45 %, (b) AS 100 S 15 % (w/w) represents 8.5 %,
(c) AS 100 E 15 % (w/w) represents 15.1 % damaged surface area

6.4 Conclusion

Low haemolytic data and histological cross sections of treated excised pig corneas. indicated a good tissue acceptability of both AS types tested, which has been confirmed in vivo with confocal laser scanning microscopy in rabbit eyes. When comparing different AS types, varying in degree of polymerization and molecular weight distribution, higher concentrations or superior emulsifying properties do not necessarily increase ocular irritation. Polymer stabilized emulsions can be an appropriate alternative carrier system for highly lipophilic and poorly soluble ocular therapeutics. Hence, AS is a promising new excipient for ophthalmic formulations due to satisfactory solubilizing and emulsifying properties coupled with a good eye tolerance.

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Part III

Biopharmaceutical aspects

7 General biopharmaceutical aspects in ocular therapy

7.1 The ocular application route and bioavailability of ophthalmic drugs

Topical administration is the route of choice for treatment of ophthalmic diseases due to the blood-ocular barrier, which is the main obstacle in the systemic treatment. Poor penetration of many drugs into the eye limits the extent to which the few systemically available medications can be used without incurring serious systemic side effects. two main sites of the blood-ocular barrier exist, which are the blood-aqueous barrier, controlling mostly inward movement, and the blood retinal barrier, controlling movement from the retina to the blood [Velez and Whitcup, 1999]. Topically applied drugs can reach the intraocular tissues by either the corneal and/or the non-corneal (conjunctival–scleral) pathways [Hosoya et al., 2005]. Lipophilic drugs, like timolol, pilocarpine or hydrocortisone, are preferably absorbed through the corneal pathway. By contrast, comparably small and hydrophilic molecules, such as D-mannitol and inulin, favour the conjunctival-scleral route, bypassing the anterior chamber and directly accessing the posterior segments of the eye [Hosoya et al., 2005]. Therefore, the cornea even though covering only one sixth of the total (human) eyeball surface area [Greaves and Wilson, 1993], is considered to be the main pathway for drug permeation into the aqueous humour and intraocular tissues.

The permeation route through the cornea varies with the physicochemical properties of the drug molecule [Robinson, 1989]. Small uncharged lipid soluble molecules preferably take the transcellular, while the less lipophilic ones prefer the paracellular pathway. The literature also reports on the existence of carrier-mediated transcorneal drug transport in rabbit eyes, e.g. for levofloxacin and L-valyl ester of acyclovir [Kawazu et al., 1999; Anand and Mitra, 2002], and on the possible endocytosis of nanoparticles [Zimmer et al. 1991]. Despite of that for most topically applied drugs, passive diffusion along the concentration gradient, either transcellularly or paracellularly, is considered to be the main mechanism for permeation across the cornea. Other physicochemical drug properties, such as solubility, partition coefficient, molecular size and shape, charge and degree of ionization, also affect the route and rate of permeation [Greaves and Wilson, 1993]. Ocular absorption of a

drug can be enhanced substantially by increasing its lipophilic character and therefore the possibility to permeate directly through the cells by using the transcellular pathway. A rapidly penetrating drug has to possess a log octanol/buffer pH 7.65 partition coefficient greater than 1 to attain optimal penetration. However, if the partition coefficient becomes higher than log 2–3, the corneal permeability is not further increased due to the limited permeability of highly lipophilic drugs across the predominantly aqueous stroma [Schoenwald, 1993].

Figure 7-1 illustrates the different permeation and elimination routes of the eye [Hornof et al., 2005].

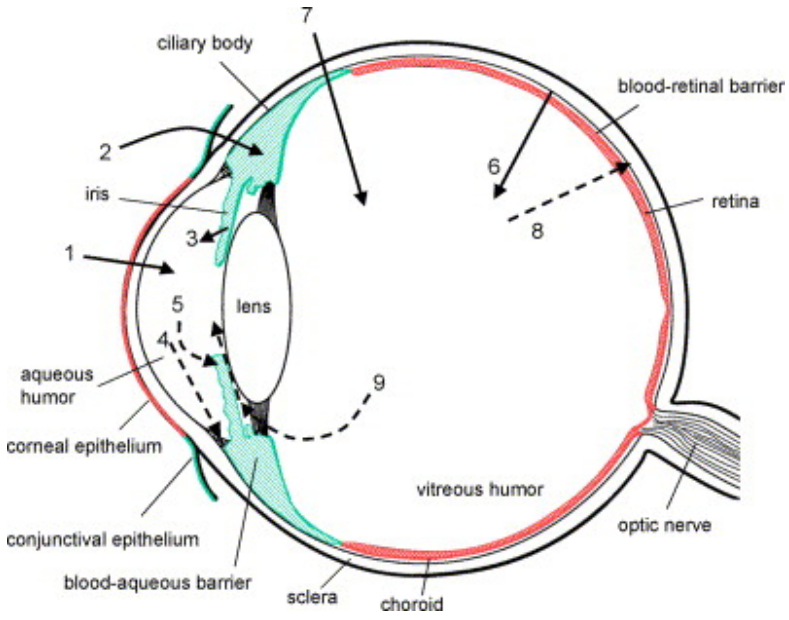

Fig. 7-1: Biological barriers, penetration (⟶) and elimination (---►) routes of the eye (tight barriers are indicated in red, others in green). (1): The main pathway for drugs to enter the anterior chamber is via the cornea. (2): Some large and hydrophilic drugs prefer the conjunctival and scleral route, and then diffuse into the ciliary body. (3): After systemic administration small compounds can diffuse from the iris blood vessels into the anterior chamber. From the anterior chamber the drugs are removed either by aqueous humour outflow (4) or by venous blood flow after diffusing across the iris surface (5). After systemic administration drugs must pass across the retinal pigment epithelium or the retinal capillary endothelium to reach the retina and vitreous humour (6). Alternatively, drugs can be administered by intravitreal injection (7). Drugs are eliminated from the vitreous via the blood–retinal barrier (8) or via diffusion into the anterior chamber (9) [adapted from Hornof et al., 2005].

The amount of drug reaching the anterior chamber of the eye depends on two competing processes: the rate of drug loss from the precorneal area and the rate of drug uptake by the cornea and conjunctiva. Topically applied drugs must encounter and compete with the essential physiological processes, like blinking, tear turnover and (basal or irritation-induced) lacrimation, protecting the eye but increasing the rate of drug loss. All three processes remove the drug and, depending on the drug delivery system, the dosage form as well. Additional mechanisms, decreasing ocular drug levels, are protein binding and/or drug metabolism [Robinson, 1989].

On the other hand for most drugs uptake by the cornea is too low. The cornea is a rather hydrophobic tight junction tissue limiting the absorption of most useful ocular therapeutics. The permeability of the corneal epithelium is rather low unless the outermost layer is damaged, either physically or by tissue inflammation. The corneal endothelium, though hydrophobic as well, is about 200 times more permeable and represents a weaker barrier [Greaves and Wilson, 1993]. Once the drug has penetrated into the cornea it has to diffuse through the hydrophilic stroma. This means that well-absorbed drugs have a mixed hydrophilic/hydrophobic nature with an intermediate partition coefficient. For a number of highly lipophilic drugs stroma and epithelium pose more difficulties and they tend to accumulate in the corneal epithelium.

In summary, ocular drug availability is typically less than 10 % [Davies, 2000] which necessitates frequent dosing to maintain therapeutic drug levels.

7.2 Optimization of ocular drug bioavailability

One of the main problems of ophthalmic therapy is the maintenance of a therapeutic drug level in eye tissues and fluids for a prolonged time period, either through a longer contact time of the ocular dosage form or medication at the eye surface or slower drug elimination. Conventional eye-drop solutions, which account for 90 % of the currently marketed formulations, consist of a specifically formulated aqueous solution, which acts as the drug vehicle. Corneal drug penetration can be enhanced by directly increasing the drug concentration. This can also be reached by increasing

the precorneal reservoir/tear film thickness with viscolysers (e.g. cellulose-ethers, polyvinylpyrrolidone and polyvinylalcohol), the application of an immiscible ocular ointment or in-situ activated gel-forming systems [Rozier et al., 1989; Balasubramaniam et al., 2003]. The two former measures are often unsatisfactory for the patient due to changes in visual acuity or blurring. Different alternative strategies, the addition of ocular penetration enhancers or mucoadhesive excipients and the application of ocular inserts, will be discussed in the following paragraphs.

7.2.1 Ocular penetration enhancers

Ocular penetration enhancers, also known as “absorption promoters”, are chemicals that modify corneal epithelium integrity and thus promote corneal drug penetration and bioavailability [Furrer et al. 2002]. At first they where used as percutaneous penetration enhancers but many reports in literature show a clear effect on the corneal tissue. The permeability of beta-blockers in rabbit eye [Sasaki et al. 1995] or the systemic delivery of insulin could be improved [Yamamoto et al., 1989; Sasaki et al. 1994].

The substances applied are mostly amphiphilic/surface active agents, such as polyoxyethylene glycol lauryl (-stearyl, oleyl) ether (Brij® 35, 78, 98), sodium glycocholate, sodium deoxycholate [Yamamoto et al., 1989], saponin, EDTA and ophthalmic preservatives like benzalkonium chloride or paraben [Sasaki et al., 2000]. While dimethyl sulfoxide (DMSO) or bile salts show a relatively good ocular acceptability, unfortunately some agents, like saponin, cause transient irritation or irreversible damage to the eye tissues [Furrer et al., 2002].

7.2.2 Mucoadhesive formulations

7.2.2.1 Bioadhesion and mucoadhesion

The term “bioadhesion” commonly refers to the adhesion between two materials where at least one of the materials is a biological substrate. In a bioadhesive drug delivery system the excipient(s) of the formulation attach to a biological tissue. In case the formulation interacts with the mucus layer that covers a mucosal tissue, the term “mucoadhesion” is employed [Robinson, 1989; Edsman and Hägerström, 2005].

In general, the use of mucoadhesive drug delivery systems offers the advantage of enhanced drug bioavailability, for both local and systemic effects, due to a prolonged formulation contact time at the site of absorption [Chowdary and Srinivasa Rao, 2004; Edsman and Hägerström, 2005]. Combined with a controlled drug release, the application of such systems improves the patient’s compliance by reducing the application frequency [Chowdary and Srinivasa Rao, 2004; Edsman and Hägerström, 2005].

7.2.2.2 Structural features of the mucous layer and mucin

The mucosa or mucous membrane is a moist tissue that lines particular organs and body cavities, such as nose, gastrointesinal tract and airways. Potential mucoadhesive drug delivery systems can therefore be developed for the buccal, oral, rectal, vaginal, nasal or ocular routes of application.

The mucosa consists of the epithelium itself and the supporting connective tissue, the lamina propria, directly underneath. Deeper connective tissue which supports the mucosa is called submucosa [Chowdary and Srinivasa Rao, 2004; Edsman and Hägerström, 2005]. In single-layered epithelia, as in the intestine and bronchi, there are non-specialized and specialized (goblet cells) epithelial cells that secrete mucus directly onto the epithelial surface. Multi-layered stratified epithelia, like in the mouth and cornea, contain or are adjacent to tissue with specialized glands secreting mucus gel. The mucus’ primary function is to mediate the interactions between epithelial cells and their environment, by means of lubrication, maintaining the water

balance, binding particles and micro-organisms or taking part in the immune response [Chowdary and Srinivasa Rao, 2004; Edsman and Hägerström, 2005].

Mucus, which refers to the total secretion from the mucous membrane, is mainly composed of mucin glycoproteins and lipids (0.5–5 %), water (95 %) and mineral salts (0.5–1 %). Further components are free proteins, enzymes and mucopolysaccharides. Mucins, which are the primary component, are a family of glycoproteins with a molecular weight of 1–40 million Da. The molecule is composed of a protein core, usually with a high serine and threonine content, and numerous O-glycosidic-linked oligosaccharide side chains bound to the core in a “bottle-brush” arrangement. There are two forms: soluble secretory and membrane bound mucin. Secretory mucins form viscoelastic gels due to their high molecular weights and ability to form complexes through intermolecular disulfide bridges, hydrophobic interactions and physical entanglement [Greaves and Wilson, 1993; Ceulemans2002a, Edsman and Hägerström, 2005]. Gel-forming mucins (MUC2, MUC5AC, MUC5B and MUC6) are responsible for the rheological properties of mucus and belong to the largest glycoproteins known. Due to the presence of sialic acid and sulphate residues the glycoprotein is negatively charged at physiological pH [Ceulemans, 2002a]. Therefore mucin can be viewed as a polyelectrolyte with a high charge density capable of holding 30–50 times its weight of water [Greaves and Wilson, 1993].

In the eye, mucus is the predominant component of the ocular tear film, maintaining a wet and healthy surface. It is primarily composed of secretory mucin, MUC5AC, secreted from conjunctival goblet cells, while membrane-associated mucins, MUC1, MUC4 and MUC 16, are present on corneal (squamous) and conjunctival epithelial cells [Ellingham et al., 1999, Aristoteli and Willcox, 2003, Gipson, 2004]. MUC7, a small soluble mucin, is expressed by the lacrimal gland acini [Gipson, 2004]. Ocular mucin is polydisperse, consisting of species with different size, charge, and glycosylation patterns that contribute to the structure and function of mucus [Berry et al., 1996; Aristoteli and Willcox, 2003].

7.2.2.3 Principles of mucoadhesion

The theories (adsorption, diffusion, electronic and wetting theory) that have been presented in connection with bioadhesion are based on previously developed theories explaining the adhesive performance of adhesives, glues and paints. They can be easily extended to describe the bioadhesion and mucoadhesion of polymeric materials to biological surfaces, respectively. According to the adsorption theory, the formulation adheres to the mucosa as a result of secondary chemical bonds, such as van der Waals forces, hydrophobic interactions, electrostatic attractions and hydrogen bonds. In the diffusion theory the polymer chains of the formulation diffuse into the mucus network and vice versa to form a semi-permanent adhesive bond through chain entanglements in the penetration region. The electronic theory assumes that an electron transfer develops from the contact between the polymer of the formulation and the mucus due to differences in their electronic structure. Adhesion occurs because of attractive forces across the electrical double layer formed at the interface.

The wetting theory was developed for liquid preparations using the interfacial tension to predict spreading and adhesion. The work done in an adhesive bond can be calculated from the measured surface and interfacial tension [Edsman and Hägerström, 2005]. Of course several of these theories must be combined to explain the process of mucoadhesion of particular pharmaceutical excipients and formulations [Edsman and Hägerström, 2005], which follows the same pattern: after establishment of an intimate contact between the mucoadhesive formulation and the mucus layer, the polymer chains and the mucin network interpenetrate to finally form bonds depending on their chemical nature. Main structural properties, which enable the interpenetration of a polymer and a biological substrate, are for the polymer having sufficient polymer chain mobility and flexibility as well as a good solubility within the mucus layer, and for both the polymer and the biological substrate having a similar chemical network structure which is sufficiently open.

7.2.2.4 Factors influencing mucoadhesion

There are many factors that can affect or influence the mucoadhesive interaction process. First of all mucoadhesion depends on the mucin type [Rossi et al., 1995] and mucin turnover [Lee et al., 2000]. Polymer-related factors are concentration, viscosity, molecular weight, degree of cross-linking, swelling and hydration [Mikos and Peppas, 1986; Junginger, 1991]. Further important factors are the pH [Lee et al., 2000], ionic strength and electrolyte concentration [Rossi et al., 1995] of the medium and contact/swelling time of the formulation [Lee et al., 2000].
Thinking of the mechanisms stated to be involved in the bioadhesion process, there are many factors, related to the mucin, medium, dosage form and polymer, which affect the formation of an intimate contact and interaction between the polymer and mucin. It became obvious that results in mucoadhesion studies even depend on the experimental setup or if the formulation is for instance dry or hydrated. Attributes that affect the mucoadhesive capacity of a polymer have been highly investigated and reported in the literature [Edsman and Hägerström, 2005].

There is an optimum molecular weight at which the chains are small enough to interpenetrate, but also large enough to entangle with the mucin molecules. This optimum depends on the flexibility and conformation of the polymer chain. High flexibility forwards interpenetration and in turn depends on the polymer type, concentration and density of cross-linking. Flexibility becomes smaller with increasing cross-linking density and concentrations. On the other hand at too low concentrations there will be not enough chains available for interaction [Smart et al., 1984; Edsman and Hägerström, 2005]. The chemical structure, the presence of ionisable groups and hydrophilicity of the polymer are important for the polymer-mucin interaction process. Especially the ability to form hydrogen bonds (hydroxyl, amine, sulfate and carboxyl groups) and/or electrostatic interactions, which is influenced by the (pH-dependent) charge density of the polymer, are significant polymer features [Ch'ng et al. 1985, Edsman and Hägerström, 2005]. A general conclusion which can be clearly drawn is that charged molecules show a better mucoadhesive capacity in comparison with non-ionic molecules, while when considering toxicity polyanions are preferred before polycations and carboxyl containing polymers are better than sulfated ones [Edsman and Hägerström, 2005].

Polyacrylic acid, a highly investigated polyanionic mucoadhesive polymer [Edsman et al., 1996; Ceulemans and Ludwig, 2002b], is capable of electrostatic and hydrophobic interactions, hydrogen bonding, and interdiffusion. Both the polyanionic hyaluronic acid [Lim et al., 2000] and polycarbophil [Lehr et al., 1991] show a prolonged precorneal residence time due to mucoadhesion, while the latter also offers an in-situ viscosity increase. Chitosan (polycationic) interacts with the negatively charged mucosal surface [Lehr et al., 1991, He et al., 1998]. Further mucoadhesive polyanionic polymers are carboxymethylcellulose [Bogataj et al. 2003] and polygalacturonic acid [Saettone et al., 1994].

7.2.3 Ocular inserts

Ophthalmic inserts are solid or semisolid, erodible or non-erodible, devices that are meant to be placed in the cul-de-sac to deliver an accurate drug dose over an extended time period at a comparatively slow and/or a controlled rate. This might offer the advantage of an increased patient compliance due to a reduced medication frequency and a lower incidence of visual and systemic side effects as a result of a decreased drainage into the nasal cavity. Additionally, internal ocular tissues, e.g. uveal tract or vitreous humour, can be targeted more easily by absorption via the non-corneal conjunctival-scleral penetration routes [Hosoya et al.,2005] and, with respect to traditional eye-drop formulations, the absence of water will increase the formulation's shelf life [Greaves et al., 1992; Gurtler et al., 1996].

One of the most successful ocular insert is the Ocusert® Pilo (Alza Corp., Palo Alto, USA, 1982). It is an insoluble insert used in the glaucoma treatment. The rate of pilocarpine release is controlled by two rate-limiting membranes on either side of the drug reservoir. However, the satisfactory kinetic behaviour is mainly owing to the unique solubility characteristics of pilocarpine free base and therefore other drugs have not been delivered in this system. Since experience has shown that twenty percent of all patients lose the insert without being aware of it, patients fitted with the device should be checked regularly [Habib and Attia, 1986; Greaves et al., 1992].

Further ocular inserts, like medicated contact lenses [Cendelin et al., 1994], the minidisc® or ocular therapeutic system (OTS), ophthalmic rods (= OR/Lacrisert®) [Greaves et al., 1992] and collagen shields [Le Bourlais et al., 1998], have been introduced. The application of water-insoluble but bioerodible/biodegradable [Deshpande et al., 1998] or water-soluble [Gurtler et al., 1996] polymeric delivery systems eliminates the need for removing the delivery system after drug release.

Soluble ophthalmic drug inserts (SODI), originally introduced in 1976 by Maichuk [Greaves et al., 1992] for the delivery of pilocarpine, have been used for delivery of a broad spectrum of ocular drugs. The original unit, a thin homogeneous polymer platelet, was made from ABE copolymer, which is a macromolecule consisting of acrylamide, vinylpirrolidine and ethylacrylate residues. The insert is placed in the inferior conjunctival sac, where wetted by the tear film it becomes plastic within a few seconds and can easily adapt to the curved shape of the eye globe. During the following minutes (10–15 min) the film turns into a viscous polymer mass, whereupon it becomes a polymer solution (30–60 min) [Greaves et al., 1992]. Ever since, water-soluble “medicated eye films” have been effectively developed and often investigated [Habib and Attia, 1986; Fitzgerald and Wilson, 1994] under variation of the polymers, preparation procedures, shape and handling (e.g. the new ophthalmic delivery system –NODS), of the delivery system [Greaves et al., 1992].

Alternative dry application forms have been described in the literature. Dry eye drops are freeze-dried single doses, composed of a hydrophilic preservative-free polymer solution containing active ingredients, attached to a tip of soft carrier strips [Diestelhorst et al., 1999]. Hydrophilic minitablets for ocular use, which were prepared by direct compression of powder mixtures containing mucoadhesive polyacrylic acid, demonstrated a prolonged ocular retention in vivo [Ceulemans et al., 2001, Di Colo et al., 2001; Weyenberg et al., 2004].

7.3 Experimental methods to investigate mucoadhesion and ocular residence time

Up to the present, most of information on mucoadhesive polymers has been provided by in vitro and ex vivo experiments. In ex vivo or in situ methods, isolated organs or tissues are adopted to imitate conditions in living organisms as closely as possible. In vivo techniques serve as the ultimate test for polymers that appear promising from initial screening using in vitro or ex vivo techniques. In ex vivo and in vivo methods the degree of mucoadhesion is derived from the residence time of the formulation at the site of application. Many different methods have been developed to investigate and compare various polymers in order to decide which one provides the longest residence time at the mucosal surface. Others have been employed to study the polymer-mucin interaction mechanism itself.

7.3.1 In vitro methods

The detachment force method, which is the most commonly employed in vitro test to study mucoadhesion, is based on the measurement of peel, shear or tensile stress when detaching a formulation from a mucosal surface, i.e. excised tissue or a mucus preparation, to calculate adhesion work from force-detachment curves [Lehr et al., 1992; Thermes et al., 1992; Tamburic and Craig, 1997]. Even though these measurements don't exactly reproduce the specific in vivo situation of the polymer/formulation tested, they give useful information about the strength of the combined mucoadhesive complex. There are several methods that have been developed for different kinds of formulations, like dry tablets [Duchêne and Ponchel, 1997], microspheres [Chowdary and Srinivasa Rao, 2004] or semisolid vehicles [Edsman et al. 1996].

A simple rheological method to assess the mucoadhesive properties of polymer solutions and gels [Hassan and Gallo, 1990] simulates the interpenetration layer by mixing the polymer dispersion with the mucin dispersion. A rheological response parameter, like viscosity or elasticity, is measured for the mixture and compared with the rheological parameters of the polymer and the mucin separately. If the value for

the mixture is larger than the sum of the values for the polymer and the mucin, it is supposed that an interaction, based on entanglements, conformational changes or chemical interactions has occurred to produce a change in the rheological behaviour. The method has first been described for viscosity measurements but in the meantime it has been adapted and broadly used for different kinds of formulations and rheological techniques, mainly oscillatory shear rheology, particularly assessing viscoelastic parameters [Tamburic and Craig, 1997; Ceulemans et al. 2001, Edsman and Hägerström, 2005].

Oscillatory shear rheology measurements, during which the viscoelastic sample is deformed sinusoidally, can be applied to specify whether the polymer system investigated is an entanglement network solution or a physical or chemical gel. Polymer networks can be divided into two main categories: covalently cross-linked materials (gels) and “entanglement networks”. However, quite a number of systems exist which possess some properties of both categories. These “physical gels” consist of chains “physically” cross-linked into networks, the cross-links themselves being of small but finite energy and/or lifetime. In many cases, the nature of the physical cross-links is not clearly known, often involving various forces such as coulombic, dipole-dipole, van der Waals, charge transfer, and hydrophobic and hydrogen bonding interactions [Ross-Murphy, 1995].

Covalently cross-linked networks are true macromolecules and formed by a variety of routes including cross-linking high molecular weight linear chains or step-addition polymerization of oligomeric multifunctional precursors. Their molecular weight and lifetime are both infinite [Ross-Murphy, 1995].

By contrast, entangled networks are formed by the topological interaction of polymer chains, either in melt or solution, when concentration and relative molecular mass become larger than a critical value. In this case, they behave as ‘pseudogels’ at frequencies higher (time scales shorter) than the lifetime of the topological entanglements [Ross-Murphy, 1995].

In the case of a “Dynamic Stress Sweep” (dss) the elastic (G’) and viscous (G”) modulus are measured against an increasing oscillatory stress (or strain) at a

constant radial frequency ω. A double logarithmic plot of G' and G" versus the stress can be generated and the linear viscoelastic region (LVER) can be determined. In a "Dynamic Frequency Sweep" (dfs) the elastic and viscous modulus are measured versus an increasing oscillatory angular frequency ω at a constant stress (strain) derived from the LVER. The dfs allows to determine the mechanical spectrum, where log G' and log G'' values are plotted versus log ω values.

Although the main purpose of the dss experiment is to determine the LVER, the results can provide useful information about the structural nature of the polymer network. In the case of a high molecular weight polymer dispersion, three different situations can be encountered in the dss: G' >> G'' values for a chemically cross-linked system, G' > G" values for a network consisting of secondary bonds and G' $\leq$ G'' values for a physically entangled polymer dispersion.

In a dfs chemically or physically cross-linked systems (strong and weak gels) yield G' values higher than G'' values (Fig. 7-2) with both moduli being mostly independent of the frequency (slope $\approx$ 0) [Ross-Murphy, 1995]. Owing to secondary interactions, the bonds remain fixed irrespective of the angular frequency applied. Therefore the same behaviour can be observed even at very low frequencies.

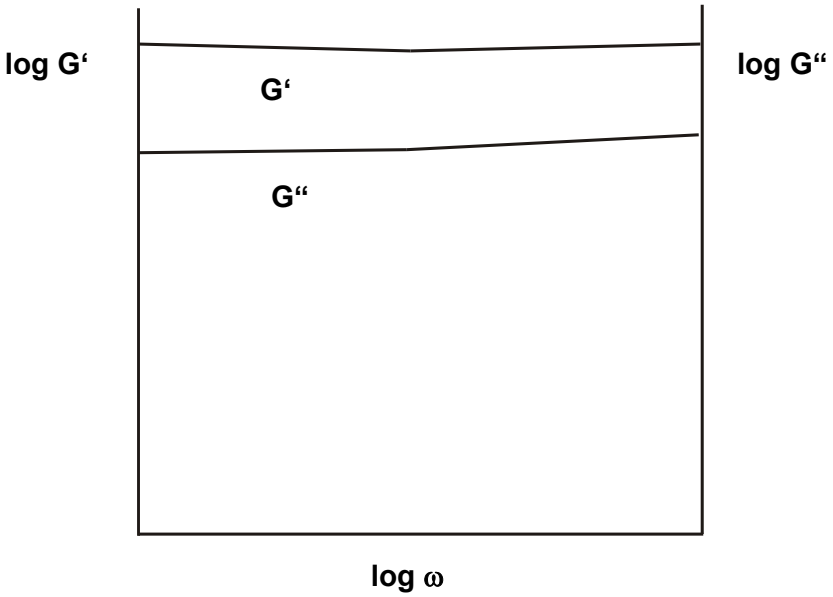

Fig. 7-2: Dynamic frequency sweep of a strong or weak gel.

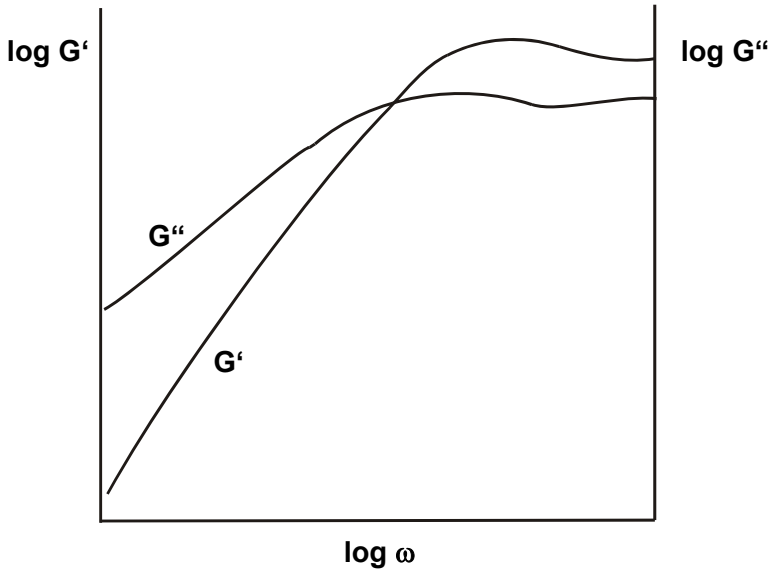

Fig. 7-3: Dynamic frequency sweep of an entangled polymer dispersion.

Entangled networks on the other hand (Fig. 7-3) result in limiting slopes at low frequency (for the elastic modulus G': slope = 2 and for the viscous modulus G'': slope = 1) in a log-log plot of moduli versus frequency while at intermediate frequencies a plateau develops [Ross-Murphy, 1995]. However, in practice due to different overlapping interaction mechanisms results may not be that straightforward and easy to interpret.

This means that in the case of interactions between mucin and the polymer, as can be drawn from a synergistic increase of viscoelasticity in the dss, the dfs results can indicate whether secondary chemical bonds or physical entanglements are responsible for these interactions. As already mentioned before, dfs measurements reveal differences between the slope values of secondary bond networks and physically entangled networks, which are most obvious in the low frequency range. The following response parameters can be considered: the slope of logG'/logω varies between 0 and 2 for respectively a perfect network of secondary bonds and a solution without any secondary bonds; the slope of logG"/logω varies between 0 and 1 under the same conditions. Therefore, the kind of interaction can be derived from the difference between the slope values of the mechanical spectra of the polymer/mucin mixture and those of the single components. If the slopes of the mechanical spectra of the polymer-mucin mixture are smaller (closer to zero) than

the corresponding slopes of the mechanical spectra of the polymer and the mucin dispersion, the formation of additional secondary bonds after mixing the polymer dispersion and mucin can be confirmed [Ceulemans et al., 2001].

Results, leading to the evidence of existent/inexistend polymer-mucin interactions, are not always easy to interpret and depend on the concentration of both the polymer and mucin, on the mucin type used [Madsen et al., 1998] and on several factors (pH, formulation, hydration state of the polymer, etc.) as noted above.

In ocular mucoadhesion experiments by means of oscillatory rheology, porcine gastric mucin is applied most commonly. Ocular mucins are not commercially available but it was found that typical properties of ocular epithelial mucins, being responsible for the mucoadhesive interaction process, are in common with those found in gastric mucin [Argüeso and Gipson, 2001]. Characteristics that support the use of gastric mucins are that ocular mucins show an extensive sialylation and sulfatation of the carbohydrate side chains. Firstly, both sialic acid and sulfate groups are generally considered as the structural characteristic being responsible for the mucoadhesive interaction observed with gastric mucin [Argüeso and Gipson, 2001; Ceulemans 2002b]. Secondly, the rheological behaviour of ocular mucin is primarily determined by the gel-forming MUC5AC mucin, which is also responsible for the rheological behaviour of gastric mucin [Argüeso and Gipson, 2001; Ceulemans 2002b].

When polymer dispersions intended for ocular use are applied topically as low-viscous preparations, the polymer molecules are fully hydrated. Since the water transfer from the mucus to the applied dosage form can be an important factor in the mucoadhesion process, as the adhesive and cohesive nature of the mucus gel increases when the water content decreases, the hydration state of the mucoadhesive polymer has to be considered in order to evaluate the effectiveness of the mucoadhesive mechanism [Edsman and Hägerström, 2005].

Another parameter influencing the mucoadhesive interaction is the concentration of the polymer incorporated in the preparation. The polymer concentration should be sufficiently high to allow a significant interaction with the glycoproteins in the mucus

layer. By contrast, increasing the polymer concentration results in an increase of the viscosity, which may cause discomfort to the patient. The bonds between the polymer and mucin must be strong enough to resist the exceptionally high shear rates present in the eye.

Furthermore, the strategy of mucoadhesion of ocular dosage forms will only be efficient if drug molecules are effectively retained in the precorneal area and the time course of the release of the drug from the mucoadhesive formulation and permeation into the corneal and conjunctival tissue matches the formulation's increased retention time on the ocular surface.

7.3.2 In vivo methods

In vivo experiments, precorneal clearance or residence time studies, constitute the ultimate testing of an in vitro approved mucoadhesive formulation, as the physiological conditions may always alter the adhesive characteristics of the material tested.

The main ocular in vivo technique to evaluate a mucoadhesive formulation is gamma-scintigraphy, e.g. in rabbits [Durrani et al., 1995; Felt et al., 1999; Chowdary and Srinivasa Rao, 2004]. This technique allows studying the distribution and intensity of radioactivity after application of a radio-labelled ophthalmic dosage form. Elimination profiles can be compared either by measuring the time necessary for eliminating 50 % of the radioactivity or by measuring the proportion of the radioactivity remaining after a given time.

Ocular fluorophotometery is the established method in clinical and laboratory areas for evaluating the permeability of the corneal epithelium [McNamara et al., 1997], the blood-retinal barriers and the blood-aqueous barrier. By measuring the concentration profile of the tracer sodium fluorescein within the anterior eye segment, after instillation or injection of a sodium fluorescein solution, the dynamics of intraocular diffusion and elimination can be accurately observed. It is a non-invasive method causing minimal disturbance to normal physiological functions. The results provide

information about the physiological and pathological state of the eye, e.g. with diabetic retinopathy, and permit the detection of physiological changes and progress in medical treatment.

Meanwhile, as reported in the literature, fluorophotometry has been successfully adopted to study the in vivo tearfilm/cornea and anterior eye segment kinetics after application of a liquid or a solid ocular dosage form containing sodium fluorescein [Ludwig and van Ooteghem, 1989; Ceulemans et al., 2001; Lux et al., 2003]. The fluorescence decay of sodium fluorescein in the precorneal tearfilm/cornea, anterior chamber and lens can be assessed to study the applied vehicles properties to improve ocular residence time or permeation. The increase of bioavailability and the prolongation of the precorneal retention time can be due to an increased viscosity of the vehicle [Ludwig and van Ooteghem, 1989] and/or the interaction between the formulation and the mucus layer present in the tear film. The apparent sodium fluorescein concentration in the anterior chamber is a principal measure of bioavailability. The concentration decay in the tearfilm/cornea, expressed as the percentage decrease per minute during basal lacrimation, may provide useful information about the ocular residence time of the fluorescent tracer as compared to a conventional eye drop solution.

The way the slit lamp fluorophotometer works is to project a beam of blue light in the

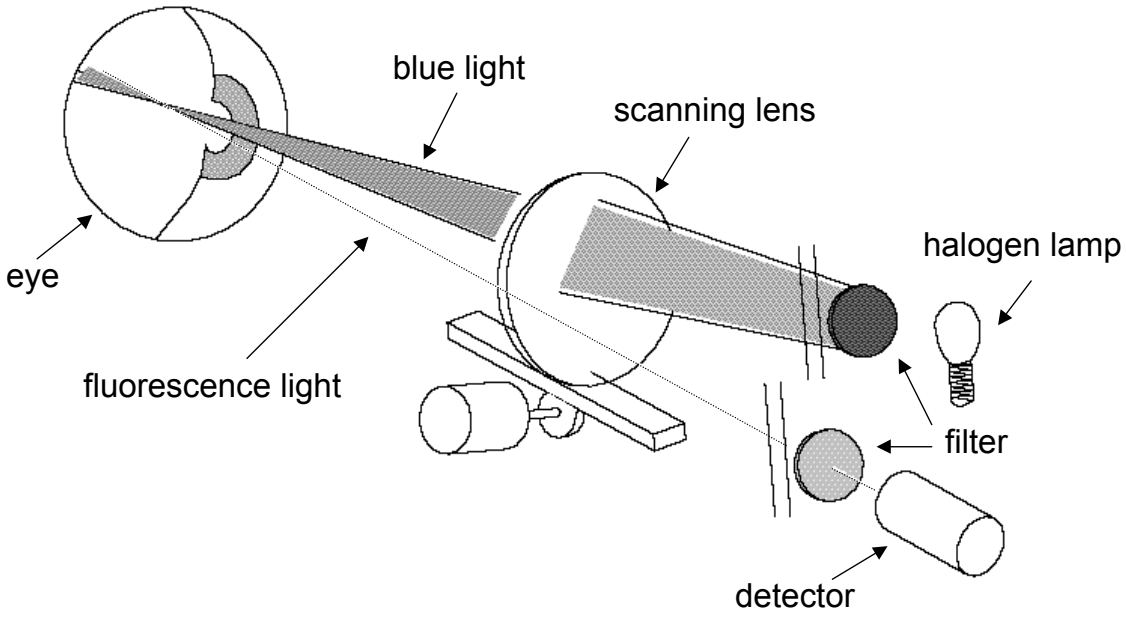

Fig. 7-4: Schematic representation of a scanning fluorophotometer (Fluorotron^{TM} Master OcuMetrics, Mountain View, CA, USA).

form of a vertical slit into the eye (Fig. 7-4). The radiation is emitted by a tungsten halogen lamp and passes through a slit and two excitation filters (transmission borders at 415 and 491 nm). At the same time, the detector, a movable scanning lens, filtered (transmission borders 532 and 630 nm) to allow only fluoresced light, is focussed on the same point in the eye. Since fluorescence can originate from each excited molecule along the excitation beam and radiates in all directions the pick up optics are also equipped with a slit, designed to gather only a slit image of the radiation originating from the focal plane of the excitation radiation.

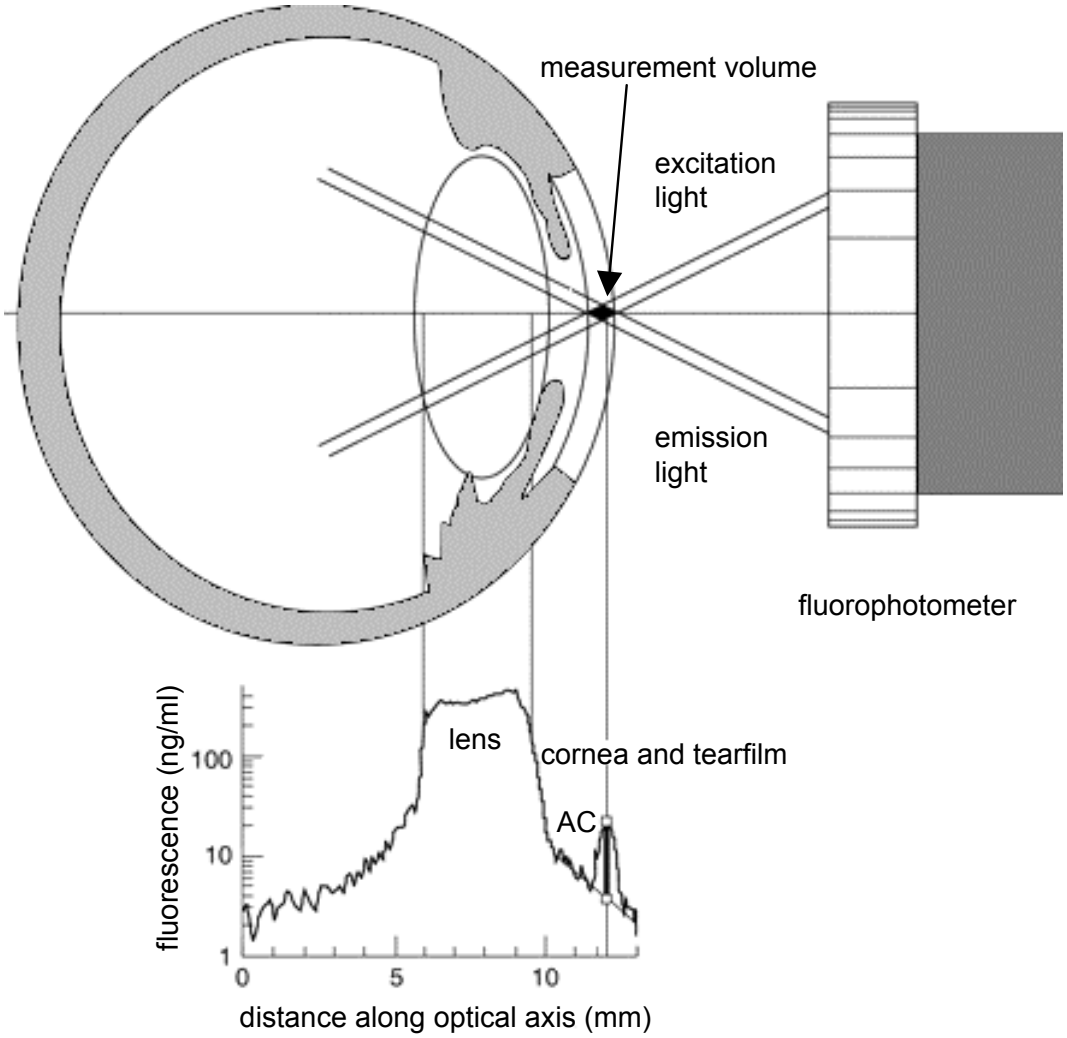

Fig. 7-5: Principle of ocular scans. Upper: a fluorescence scan of the anterior segment is obtained by moving the intersection diamond, the 'measurement volume', between excitation beam and fluorescence viewing beam along the optical axis of the eye. Lower: fluorescence profile; AC = anterior chamber [adapted from Müskens et al., 2001].

Only fluorescence at the intersection of the source light path and the detector light path is recorded and the fluorescent compound of interest is measured at only that particular point in the eye.
The apparatus moves this point of focus 149 times along the optical axis of the patient's/volunteer's eye, who has been asked to look at a fixation lamp in line with the lenses, in order to build up a profile of fluorescence (Fig. 7-5). The different positions are at a 0.25 mm distance of each other.

The signal measured by the photodetector at each scan-position is the total fluorescence of the 'measurement volume' as viewed by the detector. This volume is defined by the width and height of the excitation radiation beam, the width and height of the emission beam and by the angle of the two beams at their intersection. The dimensions of this diamond shaped measurement volume limit the spatial resolution of the fluorophotometer; only objects more than 0.5 mm removed from each other can be distinguished separately. Therefore, the cornea and the tear film response cannot be separated and are visible as a single response (Fig. 7-5). A more recently described Fluorotron model [Lux et al., 2003] seems to be more precise and allows the distinction between cornea and tear film due to a smaller measurement volume.

For a detailed scanning of the anterior segment of the eye the fluorophotometer can be equipped with an anterior chamber adapter. The magnification of this special lens is about two times larger compared to the standard lens. On a scan made with the anterior chamber adapter only the fluorescence from the first half of the eye (cornea and tear film, anterior chamber and lens) is registered.

The tear turnover (TTO; %/min) is the percentage decrease of the fluorescein concentration in the tear film per minute due to the basal tear flow. The TTO at t_x minutes after instillation of the preparation, expressed as percent per minute, is defined as [van Best et al., 1993]:

$$TTO_{t_x} = 100 \cdot \left(\frac{C_{t_x} - C_{t_x+1}}{C_{t_x}} \right) \quad (\%/\text{min}) \qquad \text{(Eq. 1)}$$

where C_t = concentration of fluorescein in the cornea/tear film compartment (ng/ml) at time t (min) after instillation. Assuming a monophasic decay of the tear film fluorescein with a decay constant k_t (min^{-1}):

$$C_{(t)} = C_{(0)} \cdot e^{-k_t \cdot t} \quad \text{(ng/ml)} \quad \text{(Eq. 2)}$$

one obtains:

$$TTO = 100 \cdot (1 - e^{-k_t}) \quad \text{(\%/min)} \quad \text{(Eq. 3)}$$

Further assuming that the fluorescence measured is proportional to the fluorescein concentration in the cornea/tear film compartment, k_t is equal to the decay constant as obtained by exponential regression to the data points obtained (Fig. 7-6). In case of a solution, the fluorescence decay is due to normal lacrimal drainage. However, in case of a polymer dispersion or a solid dosage form, the decay of the tracer is influenced by the elimination of the preparation (hydration of the preparation, dissolution and diffusion of the tracer out of the polymer network). Therefore, an Apparent Fluorescein TurnOver (AFTO) is defined [Ceulemans et al., 2001]:

$$AFTO = 100 \cdot (1 - e^{-k_e}) \quad \text{(\%/min)} \quad \text{(Eq. 4)}$$

Where k_e = apparent elimination coefficient (min^{-1}) of the monophasic decay of the cornea/tear film fluorescein concentration after application of a preparation. The first three measuring points of the fluorescence decay curve, which are influenced by reflex blinking and tearing, are excluded to calculate k_e and AFTO (Fig. 7-6).

However, some factors could influence the response parameters used, which might result in both false positive (a polymer or formulation can increase the precorneal residence time of a liquid formulation without showing a significant interaction with the mucus layer) or false negative (the precorneal residence time of the polymer is

prolonged, but the tracer is still drained rapidly through the meshes of the polymer network) results [Ceulemans et al., 2001].

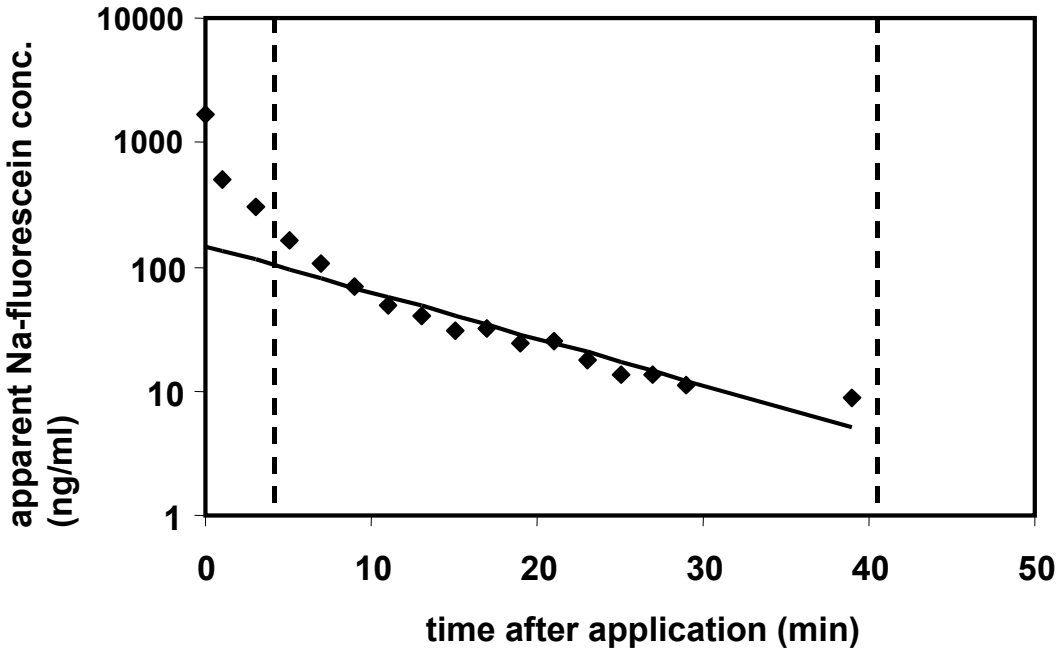

Fig. 7-6: Determination of the AFTO (apparent tear turnover). Only data points between the dashed vertical lines are used for tear turnover determination (monophasic decay) [Ceulemans 2002a].

8 Influence of *n*-octenylsuccinate starch on in vitro permeation of sodium diclofenac across excised porcine cornea in comparison to Voltaren ophtha

Published in: **Baydoun L, Müller-Goymann CC**. **2003**. *Influence of n-octenylsuccinate starch on in vitro permeation of sodium diclofenac across excised porcine cornea in comparison to Voltaren ophtha*. Eur J Pharm Biopharm 56 (1): 73–9

8.1 Introduction

Non-steroidal, anti-inflammatory drugs (NSAIDs) have proven to be a good alternative to topical steroids in the treatment of ocular inflammation. Undesired effects of steroids to the eyes include the development of posterior subcapsular cataracts and secondary infections due to an immunosuppressive effect. Further a steroid induced elevation of intraocular pressure very often occurs which is reversible once the steroid application is ceased [Goodman and Gilman, 1996].

The activity of NSAIDs is mainly based on an inhibitory effect on the synthesis of prostaglandins. A number of NSAIDs are approved for ocular application. Flurbiprofen and suprofen are used against intraoperative miosis during cataract surgery; ketorolac is given for seasonal allergic conjunctivitis and diclofenac for post-operative inflammation [Goodman and Gilman, 1996; Agata et al., 1984; González-Peñas et al., 1998]. Ibuprofen and nepafenac have also been reported to be effective in the treatment of ocular inflammation [Pignatello et al. 2002; Ke et al, 2000].

Corneal permeation depends mainly on the drug's molecular size [Grass et al., 1988; Ahmed et al. 1986], on its oil/water partition coefficient [Flynn et al., 1974; Grass et al. 1984, 1988; Yoshida and Topliss, 1996] and its degree of ionization [Edwards and Prausnitz, 2001; Huang et al., 1983]. Ionizable acidic or basic compounds penetrate corneal epithelium mainly in their un-ionized form, which is more lipid soluble [Benson, 1974; Mitra and Mikkelson, 1988]. The rate and extent of transcorneal transport is influenced by the fraction of ionized and un-ionized molecules, which in turn depends on the pK_a of the drug and the pH of the formulation [Goskonda et al., 1999]. Since most NSAIDs are weak acids ionization at lacrimal fluid pH reduces drug penetration and permeation rates, and thus drug potency. Lowering the pH of the preparation may on one hand positively influence permeation rates and drug

stability [Backensfeld et al., 1991] but on the other hand the solubility of these substances is affected. pH decrease is additionally limited and should be adjusted within a physiologically tolerated pH range, which lies between 5.8 and 11.4 [Dolder, 1990], finding an acceptable agreement with the drug's stability optimum.

Aqueous sodium diclofenac (DfNa) solutions are chemically and physically unstable [Backensfeld et al., 1991]. Therefore the commercial product Voltaren ophtha contains polyoxyethylene-35-castor oil (POC) to solubilize 0.1 % (w/v) DfNa. According to the manufacturer the solubilizing effect is based on micellization of the drug.

As reported in the literature, DfNa solutions may also be stabilized by the formation of inclusion complexes using cyclodextrins [Backensfeld et al., 1991; Reer et al., 1994; Davies, 2000]. Owing to a weaker binding strength between DfNa and the specific cyclodextrin derivative in comparison to POC, DfNa permeation rates can be improved. Solutions with decreased pH values are even more effective due to a higher diclofenac partition coefficient [Reer et al., 1994]. In vivo experiments in rabbits have shown that ocular absorption is enhanced in presence of tramazoline, an alpha-receptor stimulant [González-Peñas et al., 1998].

Amphiphilic starch (AS) is a chemically modified waxy maize starch with both hydrophilic and lipophilic surface properties. It is gained by esterification of starch with n-octenylsuccinic acid [Wolf et al., 2001]. AS is capable of producing polymer stabilized emulsions while on the other hand it may solubilize poorly soluble drugs [Baydoun and Müller-Goymann, 2000, 2001; Gers-Barlag and Müller, 2002]. Oil-in-water emulsions have proven to be appropriate carrier systems for DfNa [Bock et al., 1994].

Ophthalmic preparations require an acceptably low toxic behaviour. As described in the literature, the ocular tolerance of amphiphilic substances can be assessed by performing haemolysis studies [Reer et al., 1994; Bock et al. 1994; Pape and Hoppe, 1990; Bock and Müller 1994; Reinhart and Bauer, 1995; Kraus et al., 1990; Jumaa and Müller, 2000; Nürnberg and Frieß, 1994] which correlate highly with the Draize test and may allow to reduce the number of animals needed for in vivo studies [Pape and Hoppe, 1990]. Reports on red blood cell studies performed with Voltaren ophtha reveal high haemolytic

activities, which indicate a very low tolerance [Reer et al., 1994, Baydoun and Müller-Goymann, 2000]. It was supposed that the haemolytic effect is caused by the surfactant POC [Reer et al., 1994].

The primary objectives of the present study were to investigate the influence of AS on corneal in vitro permeation behaviour of DfNa. Permeation studies were performed through excised porcine cornea from different AS preparations, i.e. solutions and an emulsion system. Special focus was put on the variations considering AS concentrations, pH value and presence of preservative. Data were compared to those of a POC solution, a pure buffer solution, Voltaren ophtha and Voltaren ophtha sine (not preserved). All preparations contained DfNa 0.1 % (w/v) in accordance with Voltaren ophtha.

In order to interpret previously obtained results of permeation studies [Baydoun et al., 2002], saturation concentrations of DfNa depending on solubilizer type, solubilizer concentration and pH were also determined.

Moreover an attempt was made to explain the high in vitro haemolysis data achieved with Voltaren ophtha. Besides DfNa and POC, Voltaren ophtha eye drops contain boric acid and tromethamine[1] for osmolality and pH adjustment and the preservative thimerosal (manufacturer information). POC, which is also known as Cremophor EL, is often applied in parenteral emulsions as it is well tolerated by human erythrocytes [Jumaa and Müller, 2000]. Various solutions containing different substance concentrations and combinations were screened to find the component or components combination responsible for the high haemolytic effect.

[1] The name of the marketed product following this composition is currently “Diclo CV”, while the unpreserved formulation is still referred to as Voltaren ophtha sine. Both are still provided by the manufacturer mentioned.

8.2 Materials and methods

8.2.1 Materials

DfNa was purchased from Synopharm (Barsbüttel, Germany), medium chain triglycerides (MCT 812) and tromethamine from Hüls (Witten/Ruhr, Germany), purified castor oil from Henry Lamotte (Bremen, Germany), polyoxyethylene-35-castor oil (POC, Cremophor® EL) from BASF (Ludwigshafen, Germany), sorbitol from Caesar & Loretz (Hilden, Germany), thimerosal from Synopharm (Barsbüttel, Germany), boric acid and sodium hydroxide from Merck (Darmstadt, Germany). Voltaren ophtha® and Voltaren ophtha® sine (not preserved) were provided by Novartis ophthalmics (Weßling, Germany). Sodium chloride, potassium dihydrogen phosphate and disodium hydrogen phosphate (all pro analysi), purchased from Merck (Darmstadt, Germany), were used to prepare isotonic phosphate buffer pH 7.4 (PBS 7.4) and pH 6.4 according to the German Pharmacopoeia (DAB 2001); all substances used were of analytical or pharmacopoeial grade.

Acetonitrile and acetic acid (both HPLC grade) were obtained from J.T. Baker (Deventer, the Netherlands); AS type 100, an emulsifying starch, was supplied by National Starch & Chemical (Manchester, UK); double-distilled water was used for all preparations.

8.2.2 Experimental methods

8.2.2.1 Preparation of AS formulations

All preparations investigated contained 0.1 % (w/v) DfNa and were preserved with thimerosal 0.004 % (w/v) in accordance with Voltaren ophtha. pH values were adjusted to 6.5 and 7.4 using a 0.1 N sodium hydroxide solution. Preparations were isotonized with sorbitol if necessary. The investigated POC solution contained 5 % (w/w) solubilizer.

AS dispersions were prepared at concentrations of 15 and 20 % (w/w) in double-distilled water using a magnetic stirrer. Moisture contents were previously determined by thermogravimetry (Thermal Analysis System SSC 5200, Software: MAS 5700 MA-Station Version 3.2, SSC 5200H Disk-Station Version 3.2, Version/Typ: DSC 220C, Seiko Instruments, Tokyo, Japan).

An oil-in-water (o/w) emulsion (10 % (w/w) oil phase) stabilized with 15 % (w/w) AS 100 was prepared. The oil phase consisted of MCT 812 and castor oil (1:1). AS was fully dissolved in cold water and added in a stepwise manner to the oil phase using an Ultra-Turrax (Janke & Kunkel, Staufen, Germany). The pre-emulsion was passed through a high pressure homogenizer (Niro Soavi, type: Panda, Parma, Italy) six times at room temperature applying a pressure of 400 bar. The stock emulsion (AS 100 22.5 % (w/w), and lipophilic phase 15 % (w/w)) was mixed with a DfNa solution adjusting to a drug concentration of 0.1 % (w/v) and homogenized again to achieve a submicron emulsion (D90 below 1 µm). Particle size distribution was analysed by laser diffraction (Mastersizer MS 20, Malvern, Worcs, UK) and calculated by Malvern SB 09 software using the Mie approximation. pH was adjusted to 6.5 since a breaking of the emulsion was observed at pH values higher than 7 [Baydoun and Müller-Goymann, 2000].

Osmotic activities of investigated preparations were analysed by freeze point measurements (Halbmikroosmometer, Knauer KG, Berlin, Germany; the apparatus was calibrated with a sodium chloride solution (400 mOsm/kg) and bidistilled water (0 mOsm/kg)) and vapour pressure measurements (Dampfdruckosmometer type: No 11.00, Knauer KG; the apparatus was calibrated with sorbitol solutions within the concentration range of 3.0–8.0 % (w/w); the correlation coefficient obtained was > 0.999). The osmolalities of all preparations tested in permeation experiments, excluding PBS pH 7.4, are listed in table IV.2.1.

Viscosities were determined using a capillary viscosimeter (Ubbelohde viscosimeter) at a temperature of 20 °C.

8.2.2.2 Permeation experiments through excised porcine cornea

Preparation of diffusion apparatus: Prior to the experiments the corneas were carefully removed from the freshly enucleated pig eyes by incising the sclera circularly at approximately 2 mm from the corneal rim and immediately stored in PBS 7.4. The bulbi were transported in a safely closed plastic bag to avoid dehydration at approximately 4 °C and prepared within 1 h of death. Permeation experiments were carried out with modified Franz diffusion cells (Fig. 8-1) [Franz, 1975]. The excised cornea was positioned on the receptor half-cell facing it with the endothelial surface. A small amount of silicon paste was placed on the ground glass below the cornea to prevent leakage and a polycarbonate filter TMTP 5 µm (Millipore, Eschborn, Germany) was put underneath for higher stability. The donor medium consisted of the investigated formulation while PBS 7.4 was used as receptor medium. The cells used had a diffusion area ranging from 0.1963–0.2376 cm^2 and a receptor volume varying between 5.8 and 9.2 ml. The cells were kept at 37 ºC in a water bath and the receptor solutions were vigorously stirred with magnetic stirring bars.

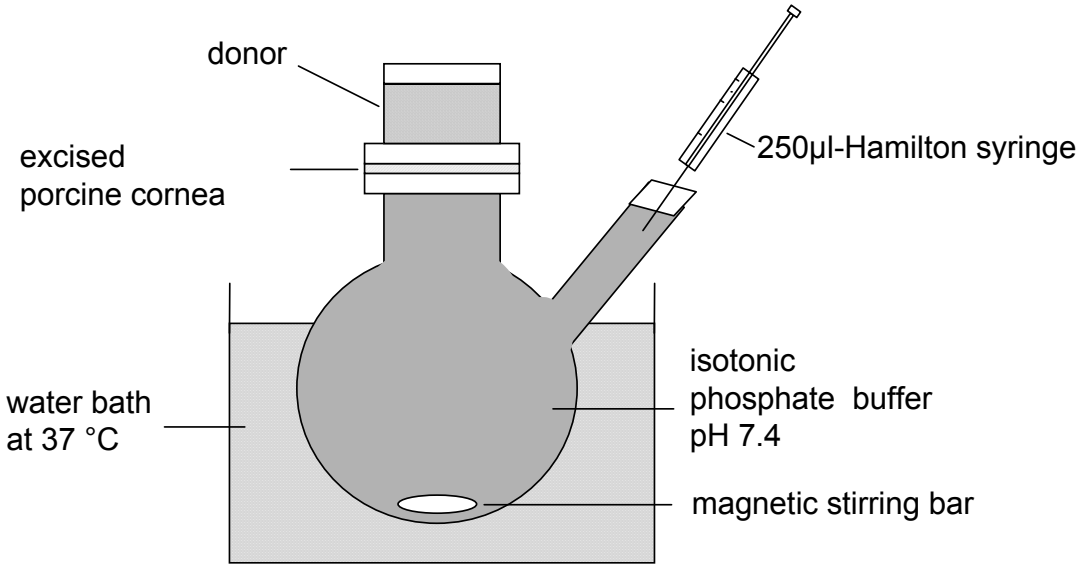

Fig. 8-1: Modified Franz diffusion cell

Samples of 250 µl were taken from the receptor compartment over 20 h to obtain correlation coefficients of 0.99 for all permeation profiles. Permeation profiles which

showed a sudden increase in permeation behaviour or correlation coefficients < 0.99 were disregarded due to a possible loss of corneal tissue integrity throughout the experiment.

The sampled volumes were replaced by fresh PBS 7.4. Permeated DfNa was analysed by UV-HPLC at 276 nm. Permeation coefficients were calculated according to Refai and Müller-Goymann (1999).

The HPLC system from Waters (Milford, MA, USA) consisted of a '486' tuneable absorbance detector, a '712 plus' autosampler and two 515 HPLC pumps. Separation was achieved with an analytical Hypersil ODS (particle size 5 µm) column (125 × 4 mm) from Grom (Herrenberg, Germany). The analytical Software Millenium 32 from Waters was used to determine the peak sizes of the permeated amounts of DfNa.

The mobile phase consisted of double-distilled water/acetonitrile/acetic acid (50/50/2). The flow rate was 1.6 ml/min and DfNa was monitored spectrophotometrically at 276 nm.

Linear correlation between peak area and DfNa concentrations was obtained within the concentration range of 0.05–25 µg/ml. The correlation coefficient was 0.999.

8.2.2.3 Red blood cell haemolysis studies

Red blood cells were obtained from human blood (27-year-old female with common blood chemistry) by centrifugation (5 min, 1000 × g). After the supernatant plasma was removed the erythrocytes were washed three times with PBS 7.4 in order to remove serum proteins. Using PBS 7.4 an erythrocyte stock dispersion with a fixed haemoglobin concentration was prepared. The stock dispersion was refrigerated and kept no longer than 24 hours.

One hundred microlitres of the stock dispersion were added to 1000 µl sample, well shaken and incubated at 37 °C for different time periods (15 min, 30 min and 1 h).

The samples were shaken every 5 min. After centrifugation (3 min, 750 × g), to remove intact erythrocytes and debris, 100 µl of the supernatant were added to 2000 µl of an ethanol/HCl mixture (40 parts of ethanol 99 % (v/v) and 1 part of hydrochloric acid 37 % (w/v)) and centrifuged again (3 min, 1000 × g). The ethanol/HCl mixture avoids haemoglobin precipitation [Jumaa and Müller, 2000].

The absorption of the subsequently achieved supernatant was measured by spectrophotometry at 398 nm against blank samples (ethanol/HCl mixtures containing the same amounts of AS and drug as the samples).

Results were set in relation to control samples of 0 % lysis in PBS 7.4 and 100 % lysis in double-distilled water. Total haemolysis must show an absorption of about 2.0 ± 0.2 in order to obtain linearity in absorption concentration dependence.

AS 100 dispersions containing 2, 4, 6, 8 and 10 % (m/m) AS with and without 0.1 % DfNa (m/v) were tested and compared to a pure DfNa solution 0.1 % (m/v) in double distilled water (0 % AS, isotonized with sorbitol, pH adjusted to 6.5) For comparison Voltaren ophtha® (0.1 % DfNa) and Voltaren ophtha sine (unpreserved, 0.1 % DfNa) were tested.

8.2.2.4 Solubility studies

To investigate saturation concentrations of DfNa, AS 100 and POC solutions with increasing solubilizer amounts were prepared (AS 100: 2, 4, 6, 8 and 10 % (w/w), POC: 0.1, 0.5, 1, 5 % (w/w)). Surplus amounts of DfNa were added to the solutions and stirred for 48 h at a temperature of 20 °C on a magnetic stirrer. After centrifugation DfNa concentrations of the supernatant were measured by UV detection at 276 nm. The studies were performed at pH 6.5 (PBS 6.5, phosphate buffer 6.4 prepared according to the German Pharmacopoeia, adjusted to pH 6.5 with HCl) and 7.4 in phosphate buffer solutions.

8.3 Results and discussion

8.3.1 In vitro permeation studies

Fig. 8-2 shows that AS dispersions seem capable of promoting corneal DfNa permeation activity when compared to permeation profiles of Voltaren ophtha.

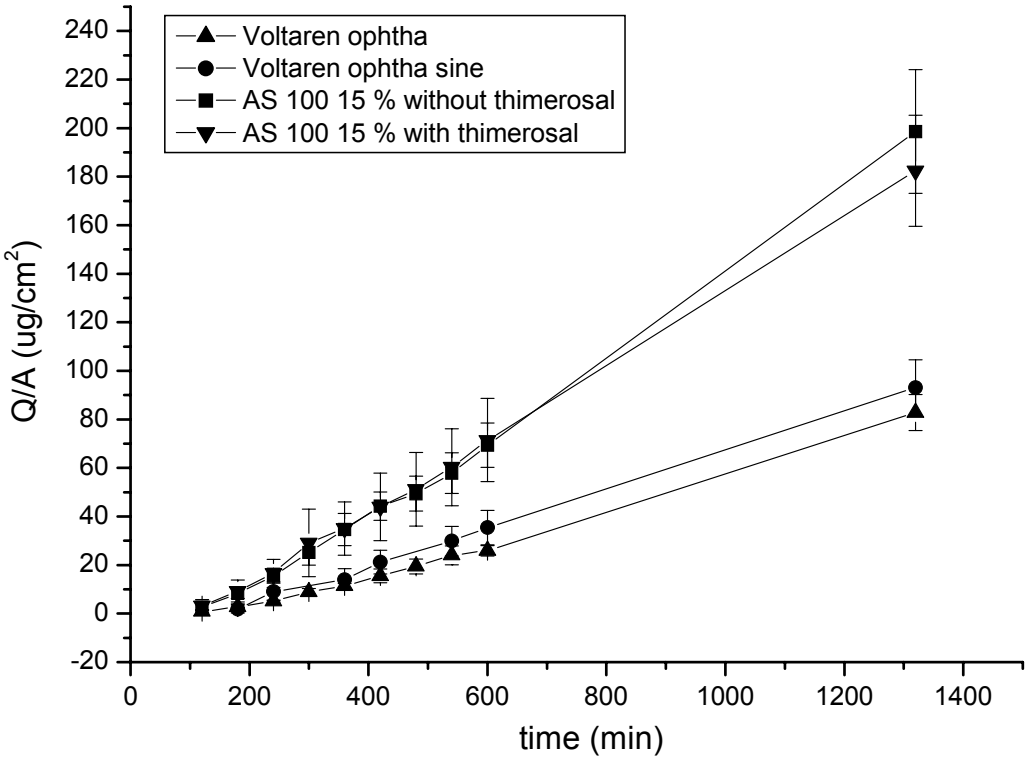

Fig. 8-2: Permeation profiles of preserved and unpreserved AS 100 15 % (w/w), pH 7.4 and Voltaren ophtha

In order to exclude a possible permeation enhancing effect caused by the preservative, dispersions with and without thimerosal were tested. As demonstrated in Fig. 8-2 and Table 8-1 showing permeation profiles and permeation coefficients, neither in the case of an AS dispersion 15 % (w/w) pH 7.4 nor in the case of Voltaren ophtha did thimerosal influence the permeation activity of DfNa. These findings are in agreement with results reported on the ocular tolerance of preservatives [Furrer et al., 1999, 2002]. In vivo experiments on murine cornea showed that a 0.01 %

thimerosal solution is not more damaging than a physiological saline solution [Furrer et al., 1999].

The permeation profiles in Fig. 8-3 reveal that the highest permeability is visibly reached with solutions of DfNa in pure phosphate buffer pH 7.4. A 15 % (w/w) AS 100 dispersion shows higher corneal permeability than Voltaren ophtha. However, permeation activity is greatly reduced with rising AS 100 concentrations, as the profile for a 20 % (w/w) AS 100 dispersion shows (Fig. 8-3). To a certain extent a higher viscosity is responsible for smaller diffusion speeds.

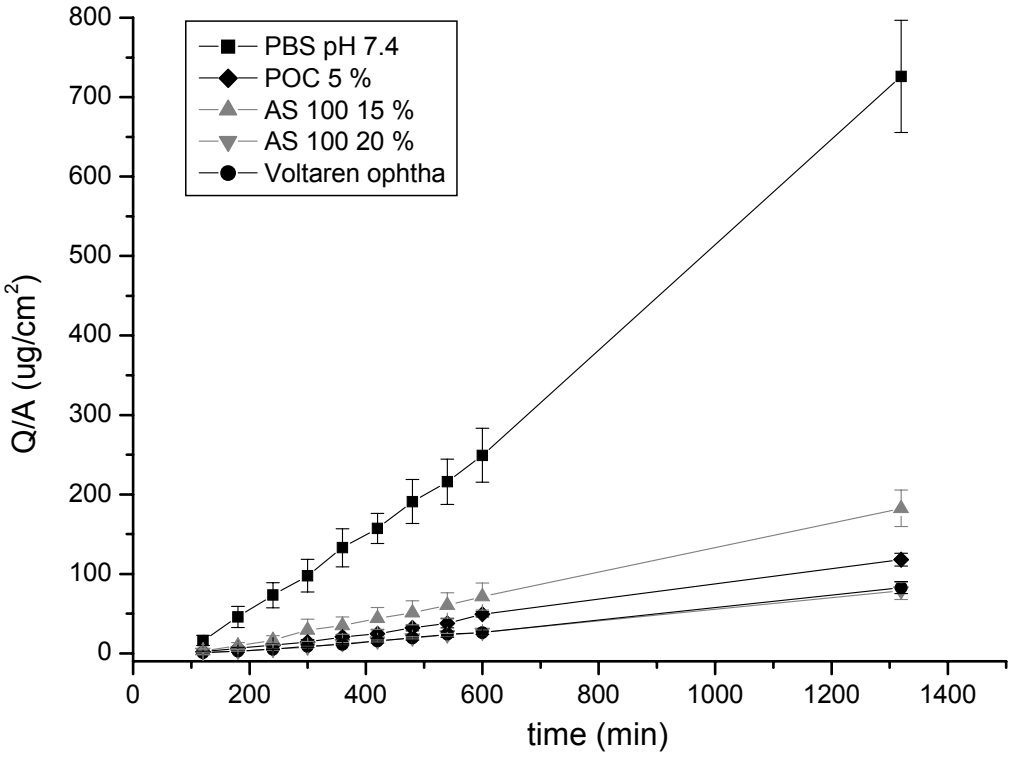

Fig. 8-3: Permeation profiles of sodium diclofenac solutions containing different amounts of solubilizer

Nevertheless, AS 100 with a concentration of 20 % (m/v), which reveals a dynamic viscosity of 48.2 mPa s, shows permeation data comparable with Voltaren ophtha or a POC solution 5 % (w/w) (Fig. 8-3). Both reveal viscosity values around 1 mPa s. This indicates that drug permeation is also influenced by interactions between drug and solubilizer.

Tab. 8-1: Permeation coefficients and osmolalities (n = 3) of different preparations containing sodium diclofenac 0.1 % (w/v), [a] each value represents mean ± SD

	Permeation coefficient [a] P (cm/s) * 10^{-6}	n	Osmolality [a] (mOsm/kg)
Voltaren ophtha	1.17 ± 0.25	9	310 ± 4.6
Voltaren ophtha sine	1.33 ± 0.15	4	308 ± 3.6
AS 100 dispersion 15 % (w/w) with thimerosal pH 7.4	2.51 ± 0.28	4	310 ± 4.0
AS 100 dispersion 15 % (w/w) without thimerosal pH 7.4	2.75 ± 0.34	4	324 ± 5.3
AS 100 dispersion 15 % (w/w) pH 6.5	2.37 ± 0.10	5	326 ± 7.1
AS 100 dispersion 20 % (w/w) pH 7.4	1.11 ± 0.10	6	433 ± 9.8
AS 100 emulsion 15 % (w/w) pH 6.5	1.24 ± 0.11	3	320 ± 5.6
POC solution 5 % (w/w) pH 7.4	1.63 ± 0.09	6	299 ± 2.5
PBS, pH 7.4	9.63 ± 0.83	3	

Solubility profiles (Fig. 8-4) point out the solubilization capacities of AS and POC at different pH values and concentrations. POC leads to comparably high saturation concentrations. AS only increases solubility at pH 6.5 while it seems to lose solubilizing qualities at pH 7.4. This agrees with the loss of emulsifying properties at pH values exceeding 7 [Baydoun and Müller-Goymann, 2000]. Another cause for reduced interactions may be a greater repulsive effect due to negatively charged octenylsuccinate side chains and also negatively charged DfNa at higher pH values.

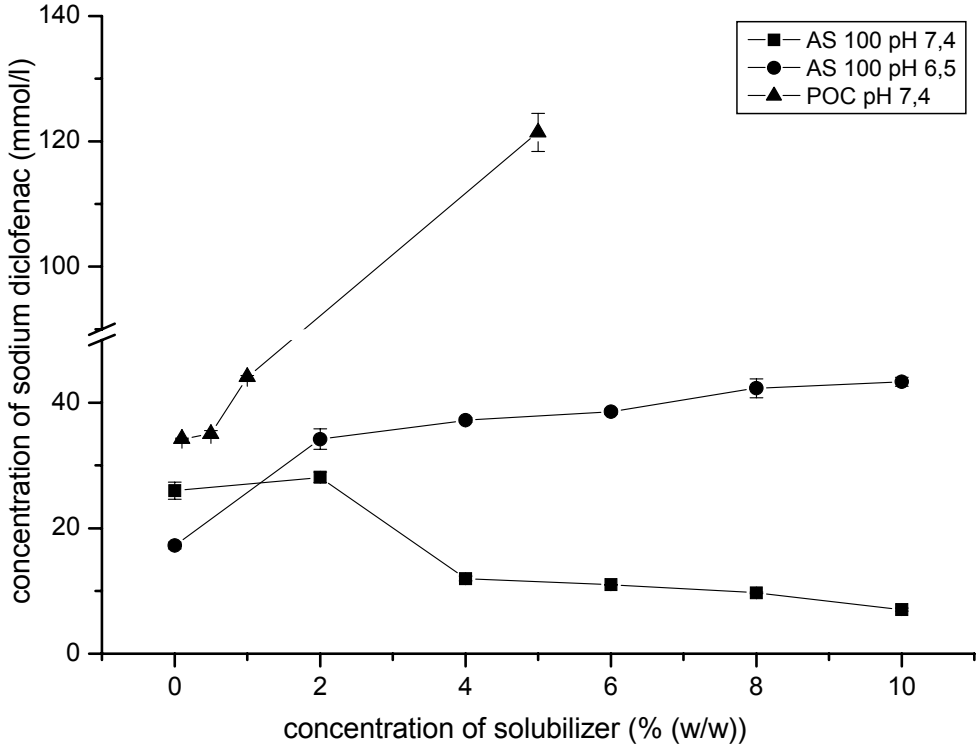

Fig. 8-4: Saturation concentrations of sodium diclofenac versus solubilizer concentration (n = 3)

Reports dealing with corneal DfNa permeation have shown that permeation data correlate with binding forces between drug and complexing or solubilizing agents [Reer et al., 1994]. Data obtained with AS also point out that permeation is generally decreased in presence of a solubilizing agent, in accordance with results achieved with complexing agents [Reer et al., 1994]. Higher absorption rates from micellar solutions can only be expected if the tenside monomers influence the structure of the epithelial cells or the tight junctions or if the drug molecules can easily leave the micelles to be absorbed.

A higher solubilizing ability, as in the case of POC, and higher solubilizer concentrations lead to diminished permeation activities. In the case of POC the solubilizing mechanism is based on a micellization of the drug while the mechanism of interaction between AS and DfNa is still unknown. AS may form inclusion compounds; however, the interactions seem to be weaker than those achieved with POC.

The permeation coefficients (Table 8-1) further show that incorporation of DfNa into an emulsion system also reduces the drug's permeation capacity. This can be explained by a higher viscosity of the system as compared to the pure starch dispersion. But since DfNa itself is an amphiphilic compound it may as well be integrated to the system's interface which can lessen the ability of DfNa to permeate through the physiological membrane due to a reduced concentration gradient of the free drug.

Although an improved permeability was expected for pH 6.5 due to a higher lipophilicity of acidic substances at lower pH values [Reer et al., 1994], the permeation coefficient does not significantly differ from that achieved at pH 7.4 [Baydoun et al., 2002]. This can be due to a pH-dependent change in the AS configuration linked with the changes in solubilising capacities (Fig. 8-4). A lower pH should promote permeation but since AS seems to be a stronger solubilizer at pH 6.5 a more rapid permeation of DfNa is hindered.

8.3.2 Haemolysis studies

Within 15 min none of the investigated AS 100 preparations including a pure 0.1 % (w/v) DfNa solution show haemolysis data higher than 1 % (Fig. 8-5). While Voltaren ophtha shows a very high haemolysis, as already reported in the literature [Reer et al., 1994, Baydoun and Müller-Goymann, 2000], which is about 33 % after 15 min (Fig. 8-5) and 87 % after 60 min (Fig. 8-6), the AS and DfNa systems' haemolytic effect remains below 1 % after 1 h (data not shown).

It was supposed that the haemolytic effect of the commercial product is caused by the surfactant contained [Reer et al., 1994] but, as indicated in Fig. 8-6, the haemolytic

activity of Voltaren ophtha becomes smaller when sorbitol 4 % (w/w) is added. This is an indication of a mere osmotic haemolysis. Additionally, POC solutions of 1, 2.5 or 5 % (w/w), isotonized with sorbitol and adjusted to pH 7.4 yield no markedly high haemolysis (Fig. 8-6). Replacing sorbitol by sodium chloride or adjusting pH with sodium hydroxide instead of tromethamine led to the same results already pointed out in Fig. 8-5 (data not shown).

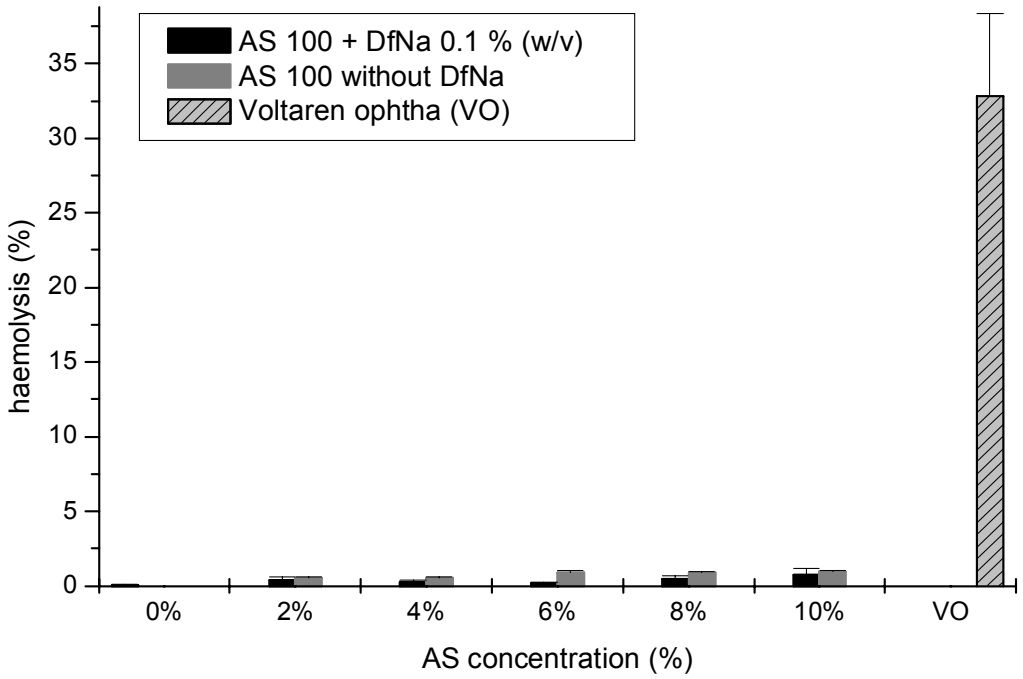

Fig. 8-5: Haemolytic activities of different AS dispersions as compared to a pure DfNa solution 0.1 % (w/v) (0 % AS) and to Voltaren ophtha after 15 min incubation at 37 ºC (n = 3)

In order to assess the component(s) in Voltaren ophtha which are responsible for the high haemolysis data, the preparations listed in Fig. 8-6 were tested. It can be seen that an isotonized POC solution (Fig. 8-6, columns 3–5) does not show significant haemolytic potential.

The apparent high haemolytic activity of boric acid (Fig. 8-6, column 1) is also lowered immensely when the solution contains sorbitol. Column 6 and 7 in Fig. 8-5 reveal the same: in the presence of sorbitol the haemolysis of a POC/boric acid mixture is diminished.

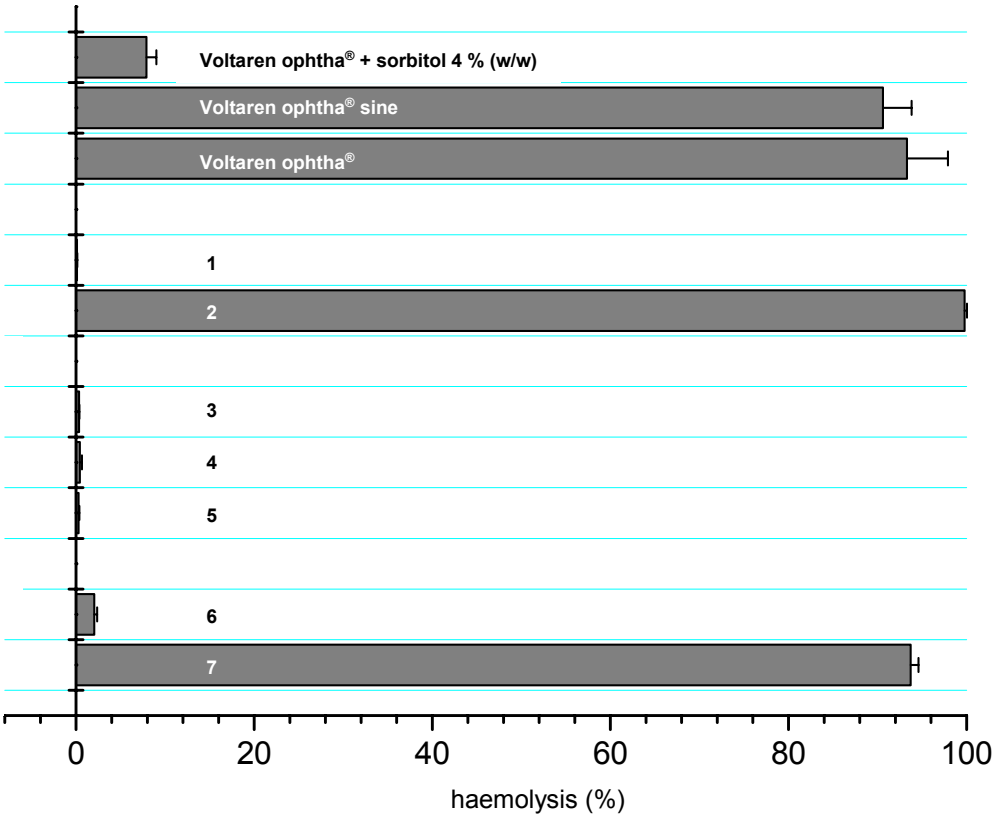

Fig. 8-6: Haemolytic activities of Voltaren ophtha, Voltaren ophtha sine and Voltaren ophtha containing sorbitol 4% (w/w) in comparison to different preparations after 60 min incubation at 37 ºC (n = 3)

1: boric acid 1.857% (w/w), sorbitol 5% (w/w), pH 7.4, adjusted with tromethamine

2: boric acid 1.857% (w/w), pH 7.4, adjusted with tromethamine

3: POC 1% (w/w), sorbitol 5% (w/w), pH 7.4, adjusted with NaOH 0.1 N

4: POC 2.5% (w/w), sorbitol 5% (w/w), pH 7.4, adjusted with NaOH 0.1 N

5: POC 5% (w/w), sorbitol 5% (w/w), pH 7.4, adjusted with NaOH 0.1 N

6: POC 5% (w/w), boric acid 1.857% (w/w), sorbitol 5% (w/w), pH 7.4, pH adjusted with NaOH 0.1 N

7: POC 5% (w/w), boric acid 1.857% (w/w), pH 7.4, adjusted with NaOH 0.1 N

Red blood cells show high haemolytic data when mixed with isotonic amounts of boric acid. This is due to the uptake of boric acid by the erythrocyte. If there is no additional isotonizer (sorbitol) the osmotic activity drops to hypotonic values, water flows into the cells followed by their breakdown. The erythrocyte membrane is not impermeable to all substances such as urea or boric acid [Jumaa and Müller, 1999]. This effect has also been described in articles dealing with parenteral emulsions. Emulsions isotonized with glycerol (2.5 % (w/w)) show high haemolytic activities

when incubated with red blood cells, while glycerol did not have any lytic properties when added to an already isotonic system [Jumaa and Müller, 1999].

8.4 Conclusion

The high in vitro haemolysis found with Voltaren ophtha is primarily based on an osmotic effect due to the erythrocyte's membrane uptake of boric acid which serves as an isotonizing substance. In this case the in vitro haemolysis test is no reliable method to evaluate the ocular irritation of Voltaren ophtha.

Haemolysis investigations respecting the toxic behaviour of the investigated DfNa-AS systems reveal that these formulations do not significantly affect the human erythrocyte.

As compared to Voltaren ophtha, AS indirectly supports the permeation activity of DfNa. It could be seen that the permeated amount depends on concentrations and solubilizing abilities of the solubilizer used. The highest permeation activity can be reached with solubilizer-free systems. Perfusion speed is greatly reduced when a solubilizer is added.

Although AS does not solubilize DfNa as efficiently as POC, it leads to higher permeation values due to a quicker release of the drug from the drug-polymer complex. Since pH, within the tested range, does not greatly influence permeation behaviour an eye drop formulation containing AS adjusted to pH 6.5 is proposed due to higher saturation concentrations reached for DfNa at this pH value.

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9 Rheological study of the *n*-octenylsuccinate starch-mucin interaction mechanism

9.1 Introduction

Divers bioadhesive drug delivery systems have been investigated to prolong the residence time of a dosage form at specific sites, which promotes drug absorption.

Together with mucin-related and medium-related factors [Rossi et al., 1995; Lee et al., 2000] various conditions and properties related to the polymer, such as concentration, viscosity, molecular weight or swelling and hydration, have been described, which support the interaction process of a polymer with the mucin layer [Mikos and Peppas, 1986; Junginger, 1991]. Several structural features of a polymer in a dosage form are known to effect the formation of intimate contact and participation in the interaction polymer-mucin process. An important chemical characteristic is the presence of ionisable groups and strong hydrogen-bonding groups (-OH, -COOH), enabling the polymer to form hydrogen bonds and/or electrostatic interactions at the polymer mucin interface [Ch'ng et al. 1985, Edsman and Hägerström, 2005]. Polycationic polymers like chitosan interact with the negatively charged mucosal surface [Lehr et al., 1991, He et al., 1998] while polymers with high surface energy properties favour the spreading onto mucus [Lehr et al., 1992].

Recently a thiolated poly(methacrylic acid)-starch composition with improved mucoadhesive properties, based on the formation of disulfide bonds between the thiolated polymer and cysteine-rich subdomains of the mucous gel, was developed and investigated [Bernkop-Schnürch et al., 2004].

Amphiphilic starch of the *n*-octenylsuccinate starch type (AS) is a chemically modified waxy maize starch gained by substituting hydrophilic starch moieties by lipophilic *n*-octenylsuccinic acid groups [Gers-Barlag and Müller, 2002; Järnström et al, 1995]. Consequently, the starch acquires emulsifying properties due to feasible formation of hydrophobic-hydrophobic interactions. Despite the surface active properties AS

displays, in vivo investigations in rabbit eyes have attested a good ocular tolerance of both AS dispersions and emulsions [Baydoun et al., 2004].

The aim of the present study was to investigate possible interaction properties of AS with mucin using oscillatory shear rheology. Dynamic stress sweeps of different AS-mucin concentration combinations have been recorded. In order to clarify if rheological synergism is based on the formation of secondary bonds dynamic frequency sweeps have been performed.

9.2 Materials and methods

9.2.1 Materials

Sorbitol was purchased from Caesar & Loretz (Hilden, Germany), mucin Type II: crude from porcine stomach from Sigma Chemicals (St. Louis, MO, USA). Sodium chloride (Federa, Brussels, Belgium) and calcium chloride, magnesium chloride and potassium chloride, sodium hydrogen carbonate, sodium dihydrogen phosphate dihydrate and disodium hydrogen phosphate dihydrate, all purchased from Merck (Darmstadt, Germany), were used to prepare Simulated Lacrimal Fluid (SLF) and isotonic phosphate buffer pH 7.4 (PBS). PBS consists of $NaH_2PO_4.2H_2O$ (4.03 g/l) and $Na_2HPO_4 \cdot 2H_2O$ (16.25 g/l). SLF consists of KCl (1.79 g/l), NaCl (6.31 g/l), $NaHCO_3$ (2.18 g/l), $CaCl_2$ (44.4 mg/l) and $MgCl_2$ (47.6 mg/l) and pH was adjusted to pH 7.4 using 1 N HCl. Purified or double-distilled water was used for all preparations. All used substances were of pharmacopoeial or reagent grade. *N*-octenylsuccinate starch (AS), AS type 100, was supplied by National Starch & Chemical (Manchester, United Kingdom).

9.2.2 Experimental methods

9.2.2.1 Preparation of tested AS and mucin dispersions for rheological measurements

An AS 100 stock dispersion containing AS 40 % (w/w) in double-distilled water, adjusted to a pH value of 6.5 using a 0.1 N NaOH solution, was prepared in advance. This polymer dispersion was stirred for about 1 h at room temperature using a magnetic stirrer and was kept in the refrigerator (6 ± 2 °C) for at least 24 h in order to achieve complete polymer hydration.

The examined AS 100 dispersions were prepared by diluting the required amount of the polymer stock dispersion with PBS adjusting AS 100 concentrations of 25, 27.5 and 30 % (w/w). Moisture contents of the starch powder were previously determined by thermogravimetry (Thermal Analysis System SSC 5200, Software: MAS 5700 MA-Station version 3.2, SSC 5200H Disk-Station Version 3.2, version/type: DSC 220C, Seiko Instruments, Tokyo, Japan).

To prepare the mucin dispersions (12, 16 and 20 % (w/w)) the required amount of mucin powder was dispersed in SLF using a magnetic stirrer. To ensure complete hydration of the mucin the dispersions were stirred for 24 h at room temperature.

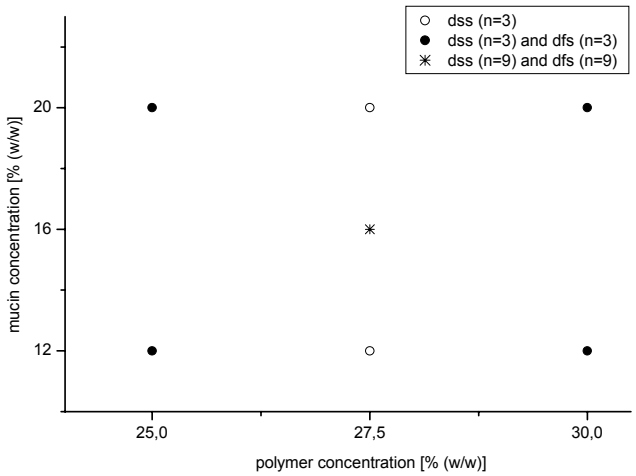

Fig. 9-1: Mucin-polymer concentration combinations tested, open circles: dss measurements (n=3); filled circles dss and dfs measurements (n=3); centre point (asterisk): dss and dfs measurements (n=9).

To analyse the degree and mechanism of interaction between AS 100 and mucin, equal amounts (w/w) of polymer dispersion and mucin dispersion were mixed reducing the concentration of both the polymer and mucin in half. This dilution procedure should as much as possible simulate in vivo conditions as obtained after instillation of an eye drop. Since, under normal circumstances, the maximum volume of the precorneal tear film is about 10 µl and the maximum volume which can be held by the eye without spilling to the cheek is 20 µl, the instillation of a 10 µl eye drop leads to a 1:1 dilution with lacrimal fluid [Robinson, 1989; Ceulemans, 2002].

In order to compare the rheological response of the polymer/mucin mixture with the rheological behaviour of the single components, equal amounts of the AS dispersion and the mucin dispersion were mixed with SLF, respectively. The polymer dispersion's rheological features are due to polymer/polymer, polymer/PBS and polymer/ SLF interactions while on the other hand mucin/mucin and mucin/SLF interactions are measured. Dynamic stress sweeps (dss) and dynamic frequency sweeps (dfs) have been performed with seven different concentration combinations, AS/mucin 25/12, 25/20, 27.5/12, 27.5/16 (centre point), 27.5/20, 30/12 and 30/20 % (w/w), as illustrated in figure 9-1.

9.2.2.2 Oscillatory shear rheology

Oscillatory shear rheology was applied to study the samples' visco-elastic properties, which were assessed by the calculation of storage moduli (G'), which represent the elastic part, and loss moduli (G''), which represent the viscous part of the studied sample. Polymer-mucin interactions, either through secondary bond formation, physical entanglement or a combination of both, can be drawn from a synergistic increase of visco-elasticity as compared to the visco-elastic properties of the single components [Ross-Murphy, 1995; Ceulemans et al., 2001].

The influence of AS on the mucin network was analysed using a Carri-Med CSL² 100 rheometer (TA Instruments, Brussels, Belgium) fitted with a double-concentric cylinder geometry. A pre-shear period (20 s, 20 1/s) was applied to disperse the preparation homogeneously in the geometry. After that the sample was left for 5 min

to rebuild and equilibrate to a measuring temperature of 32° ± 0.1 °C in accordance with the temperature at the eye surface [Morgan et al., 1995]. All rheological measurements in this study were performed at 32° ± 0.1 °C.

During a dynamic stress sweep (dss), at a constant radial frequency ω of 1 rad/s, oscillation stress was increased logarithmically. A double logarithmic plot of G' and G'' versus stress is produced to detect the linear visco-elastic region (LVER), which is characterized by a directly proportional relationship between the stress applied and the strain of the sample. From the LVER a stress value was selected to perform a dynamic frequency sweep (dfs). The performance of a dynamic frequency sweep, during which the oscillatory angular frequency ω was increased logarithmically from 0.1 to 10 rad/s, enables to clear up the exact interaction mechanism between the polymer and mucin.

9.3 Results and discussion

Dss profiles of the different AS concentrations and their influence on mucin reveal that the higher the polymer and mucin concentrations, the higher the dynamic moduli G' (Figs. 9-2, -3 and -4) and G" (Fig. 9-4) become. The greater elastic response obtained through combination with mucin 16 % (w/w) (~ 0.3 Pa) and mucin 20 % (w/w) (~ 0.2 – 0.3 Pa) as compared to mucin 12 % (w/w) is probably mainly due to the increased mucin concentration. When compared to the literature mucin concentrations tested were chosen exceptionally high for the in vitro studies in order to facilitate a rheological response, although such high concentrations can be expected directly on the corneal and conjunctival surface in vivo. However, differences in elasticity between the three AS concentrations tested and their influence on the elasticity of mucin could not be clearly exposed (Figs. 9-2, -3).

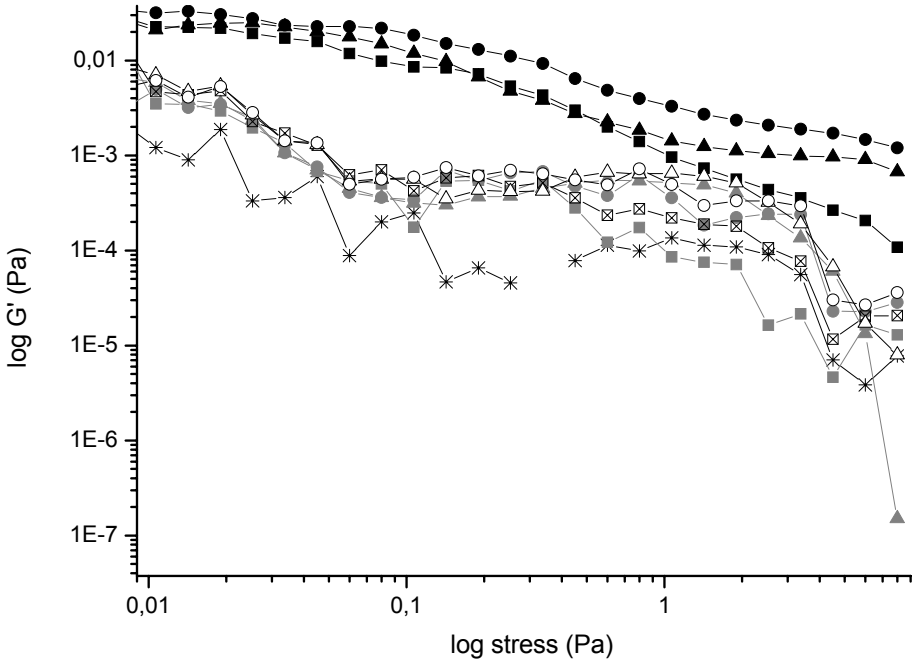

Fig. 9-2: Dynamic stress sweep illustrating the influence of AS on the viscoelasticity of mucin 12% (w/w); mean values of G‘ (n = 3); AS 25% (w/w) (squares); AS 27.5% (w/w) (triangles); AS 30% (circles); AS/SLF (grey symbols), AS/mucin (black symbols), calculated AS/mucin curves (open symbols), mucin (asterisks).

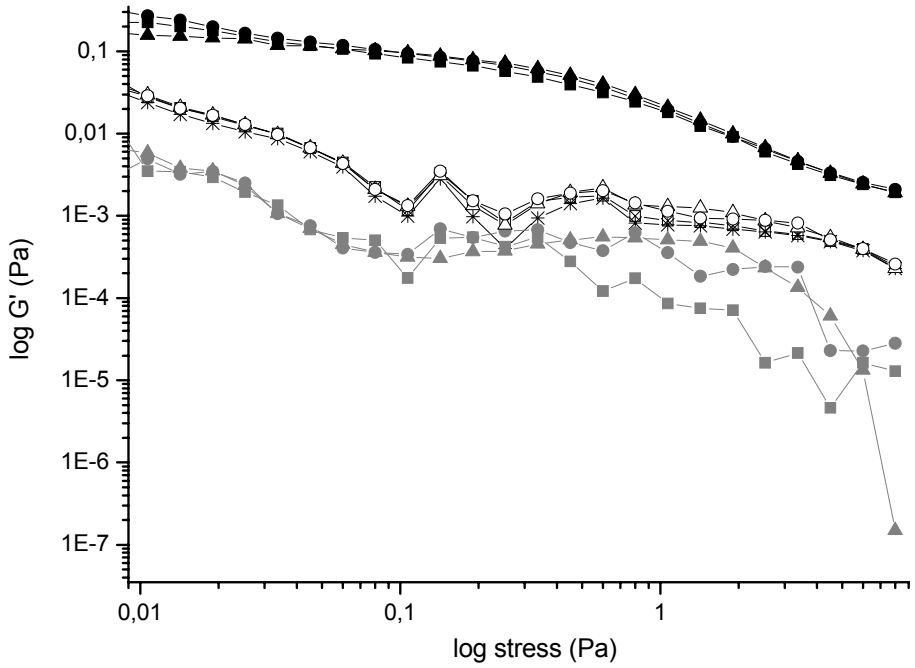

Fig. 9-3: Dynamic stress sweep illustrating the influence of AS on the viscoelasticity of mucin 20% (w/w); mean values of G‘ (n = 3); AS 25% (w/w) (squares); AS 27.5% (w/w) (triangles); AS 30% (circles); AS/SLF (grey symbols), AS/mucin (black symbols), calculated AS/mucin curves (open symbols), mucin (asterisks).

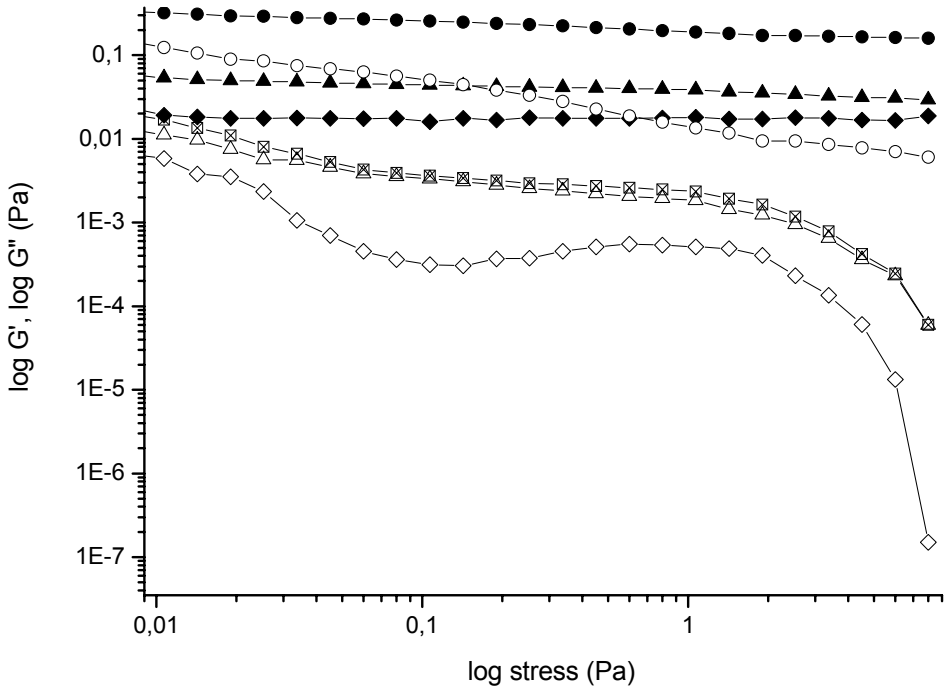

Fig. 9-4: Dynamic stress sweep illustrating the influence of AS 27.5% (w/w) on the viscoelasticity of mucin 16% (w/w); mean values (n = 9) of G‘ (open symbols) and G“ (filled symbols); mucin/SLF (triangles); AS/SLF (diamonds), AS/mucin (circles), calculated AS/mucin curve (open squares).

Curves resulting from a dss of the specific mucin/polymer mixtures reveal higher G’ values than a calculated curve resulting from the sum of the single components’ curves (Figs. 9-2, -3 and -4). This synergistic increase of elasticity is either caused by the formation of secondary bonds, physical entanglement of both components or a combination of both. The ratio G’/G” roughly indicates whether a sol (G’ < G”) to gel (G’> G”) transition occurs when mixing polymer and mucin dispersions. The polymer-mucin mixture being a gel while both the mucin-SLF and polymer-SLF dispersions being a sol could not be observed when AS 27.5 % (w/w) and mucin 16 % (w/w) were combined (Fig. 9-4), which also applies to the other concentration combinations tested (data not shown). All analysed samples behave, within the stress range applied, mainly viscous (Fig. 9-4). Therefore the formation of an elastic interaction between AS and mucin cannot be clearly documented which means that the polymer mucin mixture is not characterized by secondary bonds (G’ values smaller than G”

values). The presence of the considerable elastic response as compared to the single components points to a transient network stabilized by physical entanglement. This conclusion can be confirmed by dfs data (Tab. 9-1), which enable to clarify the characteristics of the networks analyzed.

If the slopes obtained with the different polymers and mucin-polymer mixtures are considered, it can be concluded that all dispersions tested contain molecular networks consisting of a combination of physically entangled and cross-linked chains since the slopes are larger than zero but smaller than 2 (log G‘/log ω) (tab. 9-1). The frequency dependence quantified by the slope values (tab. 9-1) points to the presence of a considerable amount of transient physical bonds between AS and mucin.

Tab. 9-1: Frequency dependence of the dispersions tested, mean values ± SD (n=3), [a](n=9).

	log G’/log ω ± SD
AS 25 % / SLF (1:1)	0.765 ± 0.15
AS 27.5 % / SLF (1:1)	0.350 ± 0.06
AS 30 % / SLF (1:1)	0.365 ± 0.08
AS 25 % / mucin 12 % (1:1)	0.770 ± 0.06
AS 25 % / mucin 20 % (1:1)	0.865 ± 0.18
AS 27.5 % / mucin 16 % (1:1)	0.588 ± 0.15[a]
AS 30 % / mucin 12 % (1:1)	0.340 ± 0.03
AS 30 % / mucin 20 % (1:1)	0.210 ± 0.08

An elastic mucoadhesive bond formation is indicated by the slope log G’/log ω for the polymer-mucin mixture being lower (less frequency dependent) than the slope values of both polymer/SLF and mucin/SLF dispersions [Ross-Murphy, 1995].

This particular behaviour is not observed for any AS concentration tested. Since the slopes, that is the frequency dependence, of the AS/mucin mixtures and the corresponding AS/SLF mixtures do not differ significantly (Student’s t-test, $P < 0.05$), no additional secondary bond formation is obtained and the increase of elasticity as indicated by the dss data is probably caused by an increase of physical entanglement between AS and mucin only. A possible explanation for the complete

absence of secondary bonds could be the negative charge of the AS molecule. Electrostatic repulsion could occur between AS and mucin, also charged negatively. On the other hand the degree of substitution (ds) with respect to ionic functions, in commercially available AS (usually 0.015) [Viswanathan, 1999], may be too low to induce electrostatic interactions with mucin.

9.4 Conclusion

Dynamic stress sweep measurements clearly indicate a rheological synergism when AS and mucin are combined. However, dynamic frequency sweeps reveal that in the case of the presented AS/mucin mixtures interactions are limited and not based on the formation of secondary bonds but on physical entanglement. Further investigations at different pH values or with respect to dry or solid AS formulations, such as powders, tablets or films, are necessary.

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10 Evaluation of different *n*-octenylsuccinate starch formulations for ophthalmic application: investigation of ocular kinetics by means of fluorophotometry

10.1 Introduction

Low ocular drug bioavailability, which results from a number of ocular anatomical and physiological constraints, which include the comparably poor permeability of the corneal epithelial membrane, the nasolacrimal drainage, blinking and tear dynamics, necessitates frequent dosing to maintain therapeutic drug levels.

The efficacy of topically administered ocular therapeutics is influenced, e.g., by the metabolism and permeation features of the drug incorporated as well as on the ophthalmic vehicle. Numerous attempts have been made to increase ocular bioavailability either by the development of prodrugs [Lee and Robinson, 1986] with an enhanced corneal penetration or by optimising the drug delivery systems with respect to a prolonged preocular residence time. Most commonly hydrogels based on natural, synthetic or semi-synthetic polymers [Ludwig et al., 1992] are applied to increase the contact time with the eye surface and reduce drug elimination. High molecular weight polymers like hyaluronic acid [Greaves and Wilson, 1993], have been shown to slow the turnover of the preocular tearfilm.

For a number of highly lipophilic therapeutics, to which the cornea is relatively permeable, the formulation of a suitable dosage form poses high difficulties. Aqueous eye drop solutions are broadly accepted by patients but do not meet the specific drug's demand for sufficient dissolution/solubilization. Surface active solubilising agents may also act as penetration enhancers, which in turn may elevate tear flow due to ocular irritation [Furrer et al.; 2002].

Oil-in-water emulsions are particularly valuable in the delivery of water-insoluble drugs. The drug, dissolved in the internal oil phase, can be kept in the preferred solution state [Ding, 1998]. In vivo data obtained from o/w-emulsions demonstrate that emulsions can be effective delivery systems [Lallemand et al., 2003] with a potential of sustained drug release [Tamilvanan and Benita, 2004]. Cationic lipid

emulsions, prepared with cationic lipids, such as stearylamine/oleylamine, or chitosan have a good spreading coefficient on the negatively charged cornea and conjunctiva due to electrostatic attraction which supports the lipophilic drug ocular disposition [Tamilvanan and Benita, 2004].

The administration of ocular inserts, developed to maintain and control drug release for a long period of time, still shows a low acceptance by both patients and physicians. To some extent this attitude can be changed by the application of soluble [Gurtler et al., 1996] or bioerodible inserts [Deshpande et al., 1998], which spare the patient the final removal of the dosage form. Soluble inserts consist of a hydrophilic and water soluble polymer. In the case of a soluble drug, soluble inserts are more suitable than a solution to control the diffusion process, while in the case of a less soluble drug, they are more capable than a suspension to control the dissolution process [Ding, 1998]. Films based on the mucoadhesive gelatin, developed for ophthalmic surgery (Gelfilm®) or drug delivery [Bonferoni et al., 2003], have been described in the literature. Employing an anionic polymer to interact with an alkaline drug, incorporated in a mucoadhesive film matrix, proved to be useful in modulating in vitro drug release profiles [Bonferoni et al., 2003].

n-octenylsuccinate starch (AS), which is also being discussed as a gelatin substitute (manufacturer information), shows excellent emulsifying [Viswanathan, 1999] and, in combination with polymers like poly(vinylchloride) or polyethylene, film forming attributes [Jane et al. 1991, Evangelista et al., 1991]. In vitro studies, on human erythrocytes [Baydoun and Müller-Goymann, 2003] and on porcine cornea, and vivo studies in rabbits have indicated a good ocular tolerance to AS [Baydoun et al., 2004, see Part II, chapter 5].

Results obtained from oscillatory rheology measurements do not allow to assign a strong polymer-mucin interaction capacity to the AS dispersions investigated in the previous paragraph (Part III, chapter 8). However, the addition of mucoadhesive polymers to ocular dosage forms may increase the residence time but not the bioavailability, if the drug is not successfully retained in the vehicle [Ceulemans et al., 2001]. Therefore, the aim of the present study was to investigate whether the

emulsifying and film forming features of AS could be deployed to develop an ocular drug delivery system with a prolonged contact time.

Ocular fluorophotometry has been utilized to assess the precorneal retention of different AS formulations, containing a fluorescent marker, in healthy male and female volunteers. The systems evaluated were an AS dispersion, an AS stabilized o/w-emulsion and a cast AS film. The systems were kept as simple as possible to put special focus on the AS and to exclude other factors that might increase ocular retention.

10.2 Materials and methods

10.2.1 Materials

Sodium fluorescein (Na-fluorescein) was purchased from Sigma (St Louis, MO, USA), medium chain triglycerides (MCT 812) from Hüls (Witten/Ruhr, Germany), sorbitol from Caesar & Loretz (Hilden, Germany), thimerosal from Synopharm (Barsbüttel, Germany), purified castor oil from Henry Lamotte (Bremen, Germany), sodium dihydrogen phosphate dihydrate and disodium hydrogen phosphate dihydrate from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate dihydrate and disodium hydrogen phosphate dihydrate were used to prepare isotonic phosphate buffer pH 7.4 (PBS). PBS consists of $NaH_2PO_4 \cdot 2H_2O$ (4.03 g/l) and $Na_2HPO_4 \cdot 2H_2O$ (16.25 g/l). *N*-octenylsuccinate starch (AS), AS type 100, was obtained from National Starch & Chemical (Manchester, United Kingdom).

Purified or double-distilled water was used for all preparations. All used substances were of pharmacopoeial or reagent grade.

10.2.2 Experimental methods

10.2.2.1 Preparation of AS formulations for in vivo measurements

An AS 100 dispersion was prepared at a concentration of 15 % (w/w) in double-distilled water using a magnetic stirrer.

An oil-in-water (o/w) emulsion (10 % (w/w) oil phase) stabilized with 15 % (w/w) AS 100 was prepared. The oil phase consisted of MCT 812 and castor oil (1:1). AS was fully dissolved in cold water and added in a stepwise manner to the oil phase using an Ultra-Turrax (Janke & Kunkel, Staufen, Germany). The pre-emulsion was passed through a high pressure homogenizer (Niro Soavi, type: Panda, Parma, Italy) six times at room temperature applying a pressure of 400 bar. Particle size distribution was analysed by laser diffraction (Mastersizer MS 20, Malvern, Worcs, UK) and calculated by Malvern SB 09 software using the Mie approximation. pH was adjusted to 6.5 since a breaking of the emulsion was observed at pH values higher than 7 [Baydoun and Müller-Goymann, 2003].

The osmotic activities of the AS dispersion and emulsion were analysed by vapour pressure measurements at 37 °C (vapour pressure osmometer type: No 11.00, Knauer KG, Berlin, Germany; the apparatus was calibrated with a sodium chloride solution (400 mOsm/kg) and bidistilled water (0 mOsm/kg)). Sorbitol was added if necessary to achieve iso-osmotic systems.

Viscosities were determined using a capillary viscosimeter (Ubbelohde viscosimeter) at a temperature of 20 °C.

Both the dispersion and the emulsion contained 0.1 % (w/v) Na-fluorescein as a fluorescent marker and were preserved with thimerosal 0.004 % (w/v). pH values of both preparations were adjusted to 6.5 using a 0.1 N sodium hydroxide solution.

AS films were prepared by casting a polymer dispersion 40 % (w/w) in double-distilled water, adjusted to pH 7 with a 0.1 N sodium hydroxide solution, into Teflon moulds (0.5 g/cm²). The polymer dispersion was stirred for 24 h on a magnetic stirrer

to ensure complete hydration. Glycerol was added, to achieve the required concentration of 1 % (w/w) in the dry product, and blended with the suspension for 2 h before casting the films. Na-fluorescein was incorporated adjusting a concentration of 2 % (w/w) in the dried material. The thickness of the films was maintained as uniform as possible by the use of dispersions containing a constant total amount of solids. Bubble-free, transparent films were obtained by drying under vacuum for approximately 48 h at room temperature to constant weight. The films were cut into small, accurately weighed (about 5 mg) pieces, wrapped in aluminium foil and kept refrigerated (6 $\pm$ 2 °C) for no longer than 7 d prior to the fluorophotometric study. The moisture content was determined using a Karl Fischer titrator (Mettler DL35, Mettler-Toledo, Switzerland) (n=3).

The Na-fluorescein concentrations of the systems were chosen according to the sensitivity of the fluorophotometer applied. Fluorescence values higher than 2000 ng/ml result in fluorescein self-absorption and counting-saturation of the fluorophotometer electronics. Fluorescence values lower than four times the corneal autoabsorption may yield irreproducible results [Van Best et al., 1993; Ceulemans et al., 2001].

AS moisture contents were previously determined by thermogravimetry (Thermal Analysis System SSC 5200, Software: MAS 5700 MA-Station Version 3.2, SSC 5200H Disk-Station Version 3.2, Version/Typ: DSC 220C, Seiko Instruments, Tokyo, Japan).

Since AS preparations cannot be sterilized by autoclaving, the starch gets decomposed and AS emulsions break, they must be prepared and kept under aseptic conditions.

10.2.2.2 Investigation of anterior eye kinetics in human volunteers

Fluorophotometry of the anterior segment of the human eye was performed in the present study to investigate the anterior eye kinetics after application of a liquid or a solid ocular dosage form containing AS and the fluorescent tracer Na-fluorescein. The apparent Na-fluorescein concentrations (ng/ml) in the anterior eye segment, i.e. the cornea/tearfilm compartment and the anterior chamber compartment, were determined at regular times after application using a Fluorotron^{TM} Master

(OcuMetrics, Mountain View, CA, USA). The apparatus was equipped with a special lens, the anterior segment adapter, for detailed scanning of corneal and tear fluorescence and is calibrated according to the literature [Ceulemans, 2002a].

The measuring protocols were those approved within the framework of a concerted action of the European Community biomedical program on ocular fluorophotometry [Van Best et al., 1993]. Four healthy volunteers (two men/two women), who were thoroughly explained the aim of the study, agreed to participate. Each preparation was applied at least three times to one volunteer, after which mean values and standard deviations were calculated.

The cornea like the lens fluoresces naturally. Corneal autofluorescence (λ_{ex} 415 nm–491nm, λ_{em} 515 nm–630 nm) is the intrinsic fluorescence that arises from endogenous fluorophores that are physiologically present in the cornea, and is considered an indicator of disease induced changes, e.g. due to open angle glaucoma or diabetes mellitus [Müskens et al., 2001]. Therefore prior to the application of each respective formulation, the autofluorescence level of the cornea/tearfilm and anterior chamber compartment was measured to adjust the values obtained for autofluorescence after application of the preparation. To exclude increased autofluorescence values by the presence of the specific AS formulations Na-fluorescein-free systems were applied to the eye of the volunteers after which the autofluorescence was determined as a function of time. Since the blank systems did not change the anterior segment autofluorescence level of any volunteer it can be assumed that changes in the fluorescence after application of the systems tested are only due to the extrinsic fluorescence of Na-fluorescein.

After recording the autofluorescence pattern, 10 µl of the liquid preparation (dispersion/emulsion) were instilled using a sterile Eppendorf pipette or one AS film (moisture content about 20 %) was applied into the lower conjunctival sac of the volunteer's right eye. At well-defined time periods, the concentration of the fluorescence tracer in the cornea/tear film and the anterior chamber was measured. In case of a liquid preparation, the tracer concentration in both compartments was determined every 2 minutes during ½ hour and once after 45 min. In the case of the films concentrations were determined every 2 minutes during ½ hour, up to 1 h every 10 min, up to 3 h every 30 min and up to 8 h each hour after application.

The Tear TurnOver (TTO; %/min), which is the percentage decrease of the fluorescein concentration in the tear film per minute due to the basal tear flow, can be calculated at a defined time after instillation of the preparation [Van Best et al., 1993] (see also .

Assuming a monophasic decay of the tear film fluorescein with a decay constant k_t (min^{-1}) and that the fluorescence measured is proportional to the fluorescein concentration in the cornea/tear film compartment, k_t is equal to the decay constant as obtained by exponential regression to the data points obtained (Figs. 10-1, -2 and -3). In case of a dispersion, the fluorescence decay is due to normal lacrimal drainage. However, in case of a polymer dispersion, polymer emulsion or an AS film, the decay of the tracer is influenced by the elimination of the preparation. Therefore, an Apparent Fluorescein TurnOver (AFTO) is defined as [Ceulemans et al., 2001]:

$$AFTO = 100 \cdot (1 - e^{-k_e}) \qquad (\%/min) \qquad (Eq.\ 1)$$

Where k_e = apparent elimination coefficient (min^{-1}) of the monophasic decay of the cornea/tear film fluorescein concentration after application of a preparation. The first three measuring points of the fluorescence decay curve, which are influenced by reflex blinking and tearing, are excluded to calculate k_e and AFTO.

Besides the measurement of the tracer concentration in the tear film, the apparent fluorescein concentration in the anterior chamber as a function of time was measured to evaluate the specific formulation's influence on the corneal permeation and on the bioavailability. The parameters used to characterize the kinetics of the preparations are C_{max} (maximum apparent fluorescein concentration achieved; ng/ml) and t_{max} (time at which C_{max} is reached; min). Area under the curve (AUC) from time 0 to the time at the last measurable concentration (AUC_{last}: dispersion and emulsion) and to 6 h (AUC_{6h}: film) was calculated according to the linear trapezoidal rule using an Origin 6.1 program.

Data were statistically compared applying the Student's t-test (unpaired samples). A probability level of 0.05 was chosen for all comparisons. Calculations were made with a Microsoft Excel 7.0 program.

10.3 Results and discussion

10.3.1 Sodium fluorescein elimination in the cornea/tear film compartment

The results of the AFTO (Eq. 1) calculations for all tested preparations (dispersion, emulsion and film) are presented in table 10-1.

The mean AFTO value for the AS dispersion is 12.79, for the AS emulsion 8.30 and for the AS film is 3.03 %/min. The AFTO data were statistically compared applying the Student's t-test (unpaired samples). A probability level of 0.05 was chosen for all comparisons. Calculations were made with an Origin 6.1 program. The comparison of the three preparations reveals significant differences ($P < 0.05$) of AFTO data between all of the three formulations investigated. On the other hand, considering each single preparation, the differences between the volunteers is not significant ($P < 0.05$).

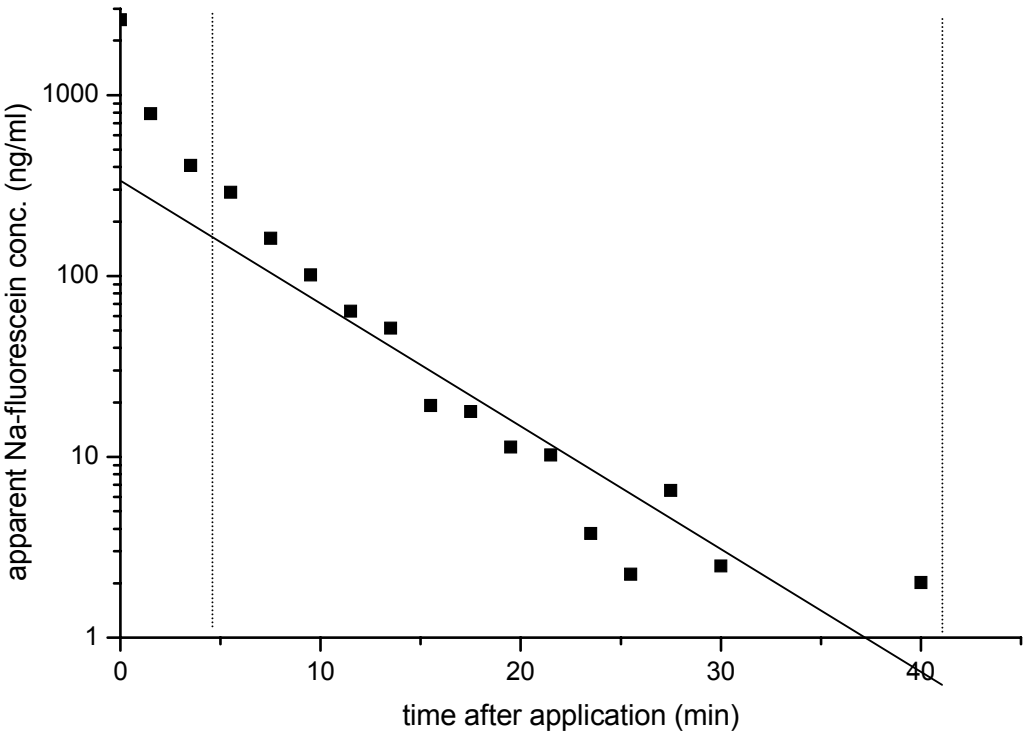

Fig. 10-1: Determination of the tear turnover (TTO) after application of an AS solution (volunteer A, TTO=14.66 %/min). Only data points between the dashed vertical lines are used for tear turnover calculation (monophasic decay).

Tab. 10-1: Mean apparent fluorescein turnover (%/min) (n = 3) ± SD after application of the reference solution (0.1 % (w/v) Na-fluorescein in PBS), the AS dispersion (15 % (w/w) AS, 0.1 % (w/v) Na-fluorescein and 0.004 % (w/v) thimerosal in double-distilled water, pH 6.5), the AS emulsion (15 % (w/w) AS, 10 % (w/w) MCT 812 and castor oil (1:1) and 0.004 % (w/v) thimerosal in double-distilled water, pH 6.5) and the AS film (2 % (w/w) Na-fluorescein, 1 % (w/w) glycerin, AS 80 % (w/w), water ad 100 %), the AFTO differences between the three preparations are significant, while, considering each single preparation, the differences between the volunteers is not significant (Student's t-test, unpaired samples, $P < 0.05$), n.m. = not measured.

Volunteer	AS dispersion	AS emulsion	AS film
A	13.46 ± 0.91	9.20 ± 0.75	2.98 ± 1.51
B	12.11 ± 1.31	7.82 ± 2.46	3.56 ± 0.25
C	n.m.	8.59 ± 0.90	n.m
D	n.m	7.60 ± 1.63	n.m

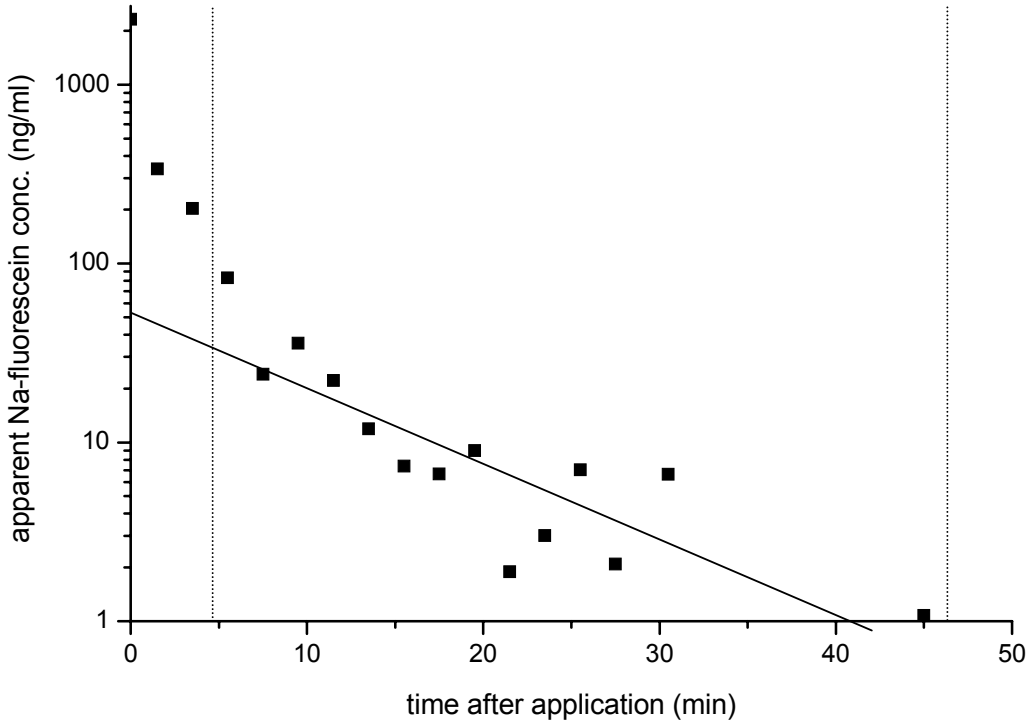

Fig. 10-2: Determination of the tear turnover (TTO) after application of an AS emulsion (volunteer A, TTO=9.25 %/min). Only data points between the dashed vertical lines are used for tear turnover calculation (monophasic decay).

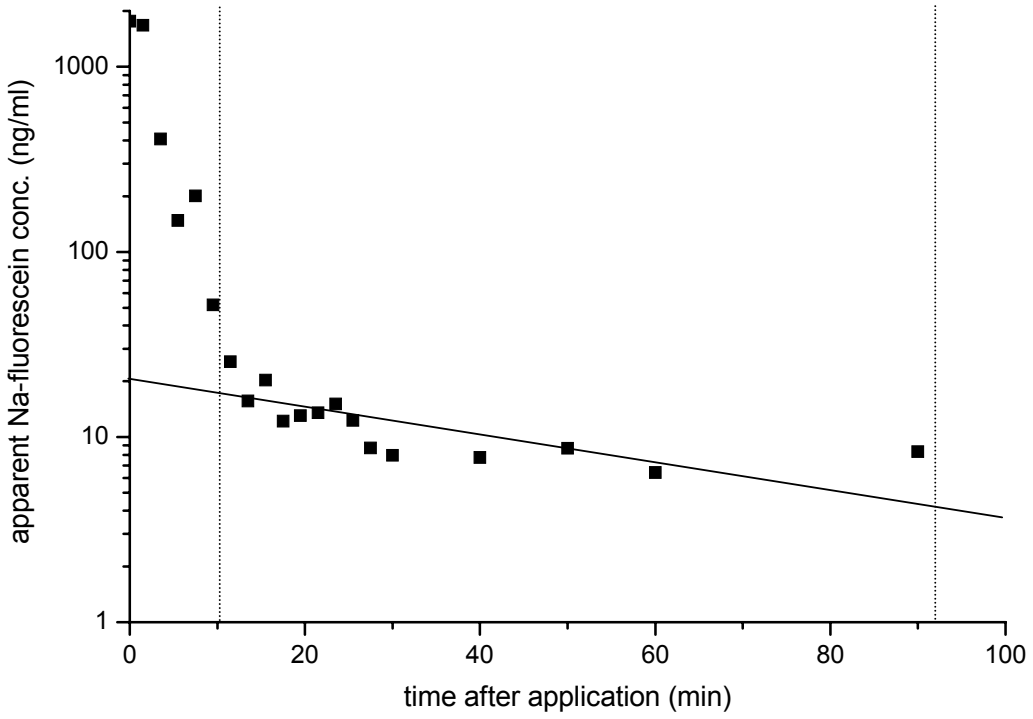

Fig. 10-3: Determination of the tear turnover (TTO) after application of an AS film (volunteer A, TTO=1.59 %/min). Only data points between the dashed vertical lines are used for tear turnover calculation (monophasic decay).

When comparing the AFTO values of the different preparations tested the most rapid elimination of Na-fluorescein from the cornea/tearfilm compartment is visible after the application of an AS dispersion (Fig. 10-1 and tab. 10-1), which is followed by the AS emulsion (Fig. 10-2 and Tab. 10-1). A significantly slower drainage from the cornea/tearfilm compartment is reached after the application of an AS film (Fig. 10-3 and Tab. 10-1).

This order was expected since, besides the lack of evidence indicating a sufficient AS-mucin interaction [Baydoun et al., 2002], the dispersion reveals a lower viscosity (15.9 mPa s) as compared to the emulsion (40.0 mPa s). Moreover, the dispersion may be less able to retain the tracer at the eye surface because Na-fluorescein can diffuse quickly out of the predominantly hydrophilic system. In contrast, the emulsion slows down the elimination. Na-fluorescein, a paracellular marker, has shown in permeation experiments across mucosal membranes that it prefers to take the paracellular path, which is the route rather hydrophilic molecules take [Clausen and

Bernkop-Schnürch, 2000]. The presence of lipophilic components (MCT and castor oil) complicates the diffusion of the, at the pH present (6.5), hydrophilic molecule.

After the application of a film the Na-fluorescein elimination rate is decreased probably on account of the film dissolving process. Firstly, the film has to take up water before it will be transformed into a high viscous solution. Diffusion rate is significantly reduced by the much higher viscosity and the closer polymer network meshes [Camber and Edman, 1989] owing to the elevated polymer concentration inside the hydrated film.

10.3.2 Sodium fluorescein elimination in the anterior chamber compartment

The mean values of the kinetic parameters, C_{max}, t_{max} and AUC, characterising the kinetic course of the apparent Na-fluorescein concentration in the anterior chamber of each volunteer are listed in the tables 10-2 and -3.

The apparent Na-fluorescein concentration in the anterior chamber is significantly increased after the application of each tested formulation (Tabs. 10-2 and 10-3). After the application of a dispersion and, respectively, an emulsion C_{max} is reached immediately after the instillation, whereas (Tab. 10-2) in the case of the films t_{max} is slightly delayed (about 1.6 min) due to the dissolving process the film has to undergo before the entire Na-fluorescein amount is released.

Tab. 10-2: Mean value ± SD of the kinetic parameters (n = 3) characterising the profile of the apparent Na-fluorescein concentration in the anterior chamber as a function of time after application of an AS dispersion and an AS emulsion; C_{max} is reached at t_{max} = 0 min, i.e. right after application; AUC_{last} = Area under the curve from time 0 to the time at the last measurable concentration, n.m. = not measured.

	AS dispersion			AS emulsion		
Volunteer	C_{max} (ng/ml)	t_{max} (min)	AUC_{last} (ng h/ml)	C_{max} (ng/ml)	t_{max} (min)	AUC_{last} (ng h/ml)
A	42,67 ± 3,10	0	1.71 ± 0.48	12.15 ± 1,96	0	0.88 ± 0.32
B	23,15 ±13,41	0	1.35 ± 1.16	20.04 ± 9.07	0	0.92 ± 0.40
C	n.m			15.13 ± 14.81	0	0.80 ± 0.21
D	n.m			56.45 ± 32.16	0	2.82 ± 0.72

Although volunteer A displays a significant decrease in C_{max} when an emulsion is applied instead of a dispersion, altogether C_{max} data of all preparations investigated do not differ significantly (Student's t-test, unpaired samples, $P < 0.05$).

Tab. 10-3: Mean value ± SD of the kinetic parameters (n = 3) characterising the profile of the apparent Na-fluorescein concentration in the anterior chamber as a function of time after application of an AS film, AUC_{6h} = Area under the curve from time 0 to 6 h, [a] n = 2.

Volunteer	C_{max} (ng/ml)	t_{max} (min)	AUC_{6h} (ng h/ml)
A	51.36 ± 22.47	1.4 ± 0,35	49.67 ± 7.69
B [a]	39.01 ± 15.50	1.73 ± 1,61	19.53 ± 1.82

However, profiles of the apparent fluorescein concentration of the anterior chamber as a function of time, considered over an extended time period, (Fig. 10-4) point out the differences between the three systems on hand.

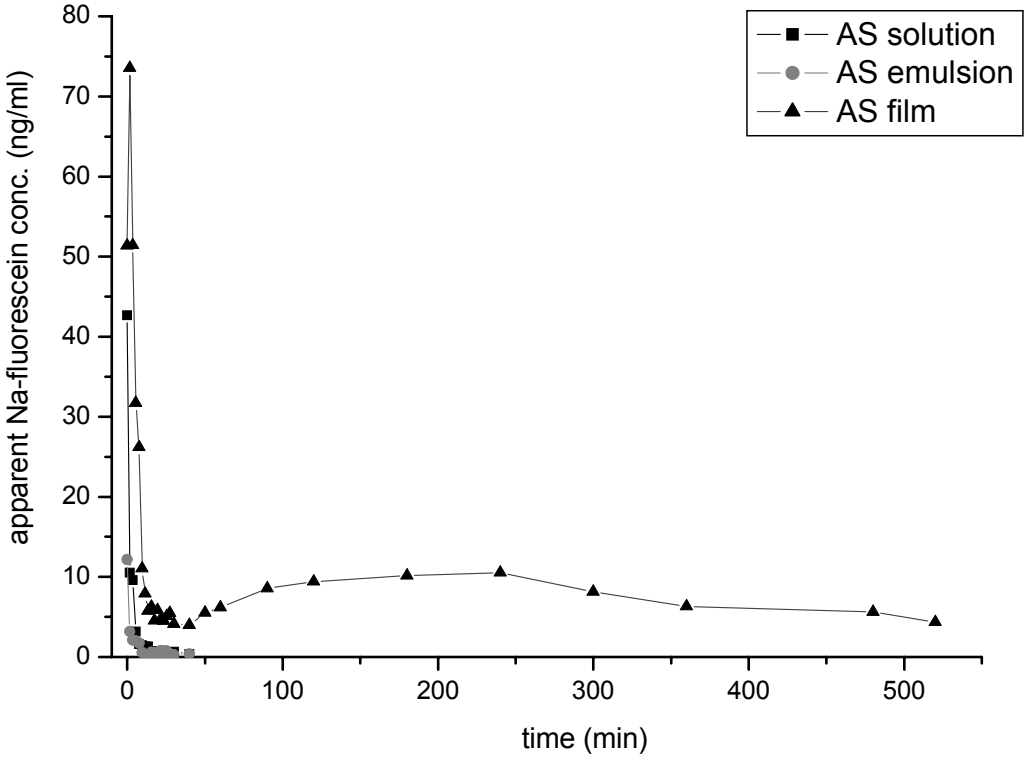

Fig. 10-4: Apparent fluorescein concentration (mean values (n=3)) of the anterior chamber compartment as a function of time after application of an AS dispersion (squares), an AS emulsion (circles) and an AS film (triangles) in volunteer A.

Relating to anterior chamber kinetics of Na-fluorescein the differences between the dispersion and the emulsion are not significant. Although the AFTO for the emulsion was improved as compared to the dispersion there is an indication on a comparable or even increased diffusion rate from the dispersion. However, in case of an amphiphilic or lipophilic drug, which greatly influences the drug's diffusion dynamics within the formulation, the results may be different. As already reported on diclofenac permeation [Baydoun and Müller-Goymann, 2003] the drug incorporation within an emulsion reduces permeation rates which, associated with the increased ocular

contact time obtained upon emulsion administration, can be useful for the development of sustained release formulations.

Although the amount of NaFLU applied with a film (100 µg after the application of a 5 mg film containing 2 % Na-fluorescein) was 10 fold larger than the amount applied with a dispersion or an emulsion (10 µg after a 10 µl-instillation of a 0.1 % Na-fluorescein solution and emulsion), comparable C_{max} values for all volunteers are obtained.

The film curve, like the dispersion and emulsion curves, drops comparably fast but while Na-fluorescein disappears within one hour after the instillation of an emulsion or dispersion, even 8 h after having applied a film Na-fluorescein was still detectable (5,62 ± 0,22 ng/ml, volunteer A) in the anterior chamber. Though not directly comparable, at similar C_{max} data, the AUC can be significantly increased after the application of a film as compared to the dispersion/emulsion (Tabs. 10-2 and -3).

Pulse entry is a common and yet highly undesirable, pharmacokinetic characteristic associated with eye drops. The initial high drug concentration found in tears followed by a rapid decline may lead to a potential risk of toxicity [Ding, 1998]. By application of an AS film drug release and corneal permeation seems to be controlled more effectively.

10.4 Conclusion

Preocular retention time of NaFLU incorporated in an AS formulation could be effectively increased following the order dispersion<emulsion<film. Nevertheless, dispersion and emulsion exhibit comparable C_{max} / AUC data in the anterior chamber, where NaFLU disappears within 1 h. AS films facilitate the application of higher NaFLU doses at a comparable C_{max} but an inevitably larger AUC due to a more controlled release and permeation. AS films may be useful in both the treatment of precorneal and intraocular diseases. In the case of poorly water-soluble drugs the AS emulsion offers a good alternative to conventional aqueous eye drops/suspensions.

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Summary and general conclusions

AS are polymers, that are capable of stabilizing emulsions without using classic emulsifying agents like surfactants. AS types that exhibit a larger molecular weight distribution may contribute more effectively to the formation of fine and stable emulsions. Emulsions with particle sizes (D90) below 1 µm and a notable long-term stability can easily be achieved.

However, AS emulsions of either tested concentration and pH break throughout autoclaving. This remained unchanged after the addition of co-emulsifiers commonly used to prepare parenteral emulsions. Some partly even prevented emulsion formation from the first due to strong interactions. Therefore AS formulations generally need to be prepared under aseptic conditions.

Since AS loses emulsifying properties at pH values above 7, emulsions should be adjusted to 6.5 to achieve an acceptable compromise between stability and ocular tolerance.

AS emulsions can easily be spray dried and reconstituted prior to usage, whereas an emulsion with a non-evaporable content of 40 % (w/w) (AS 25 % and 15 %) proved to yield stable emulsions with comparable droplet sizes as obtained prior to spray drying.

Toxicology data achieved with in vitro and in vivo measurements indicated a good tissue acceptability and eye tolerance of the AS types tested, which makes it an appropriate surface active polymer and stabilizing agent for ocular preparations.

As compared to Voltaren ophtha, AS indirectly supports the permeation activity of DfNa. Although AS does not solubilize DfNa as efficiently as POC, it leads to higher permeation values due to a quicker drug release from the drug-polymer complex. Since pH, within the tested range, does not greatly influence permeation behaviour an eye drop formulation containing AS adjusted to pH 6.5 is proposed due to higher saturation concentrations reached for DfNa at this pH value.

Although interactions with (ocular) mucin seem to be limited fluorophotometric investigations in human volunteers revealed that the preocular retention time of Na-fluorescein could be effectively increased with the application of an AS emulsion as compared to an AS dispersion, and an AS film as compared to an AS emulsion. The ocular application of soluble AS films even resulted in detectable fluorescein concentrations over an extended time period of several hours in the anterior chamber suggesting that AS films may be useful in both the treatment of precorneal (inflammation, infection) and intraocular (glaucoma, cataract, infection) diseases.

Considering conventional aqueous eye drops, AS offers good alternatives, such as emulsions and dry redispersable emulsions for poorly water-soluble drugs, AS dispersions with increased corneal DfNa permeation and AS films for controlled delivery and an enhanced ocular bioavailability. Hence, due to satisfactory solubilizing and emulsifying properties coupled with a good tissue and eye tolerance, AS is a promising new excipient for ophthalmic and other non-enteral formulations.